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Case Control Study

Comparative study of type 2 diabetes mellitus-associated gut microbiota between the Dai and Han populations

Tang LT *et al*. Gut microbiota in Han and Dai

Abstract

BACKGROUND

The global prevalence of type 2 diabetes mellitus (T2DM) is increasing. T2DM is associated with alterations of the gut microbiota, which can be affected by age, illness, and genetics. Previous studies revealed that there are discriminating microbiota compositions between the Dai and the Han populations. However, the specific gut microbiota differences between the two populations have not been elucidated.

AIM

To compare the gut microbiota differences in subjects with and without T2DM in the Dai and Han populations.

METHODS

A total of 35 subjects of the Han population (including 15 healthy children, 8 adult healthy controls, and 12 adult T2DM patients) and 32 subjects of the Dai population (including 10 healthy children, 10 adult healthy controls, and 12 adult T2DM patients) were enrolled in this study. Fasting venous blood samples were collected from all the subjects for biochemical analysis. Fecal samples were collected from all the subjects for DNA extraction and 16S rRNA sequencing, which was followed by analyses of the gut microbiota composition.

RESULTS

No significant difference in alpha diversity was observed between healthy children and adults. The diversity of gut microbiota was decreased in T2DM patients compared to the healthy adults in both the Dai and Han populations. There was a significant difference in gut microbiota between healthy children and healthy adults in the Han population with an increased abundance of Bacteroidetes and decreased Firmicutes in children. However, this difference was less in the Dai population. Significant increases in Bacteroidetes in the Han population and Proteobacteria in the Dai population and

decreases in Firmicutes in both the Han and Dai population were observed in T2DM patients compared to healthy adults. Linear discriminant analysis Effect Size analysis also showed that the gut microbiota was different between the Han and Dai populations in heathy children, adults, and T2DM patients. Four bacteria were consistently increased and two consistently decreased in the Han population compared to the Dai population.

CONCLUSION

Differences in gut microbiota were found between the Han and Dai populations. A significant increase in Bacteroidetes was related to the occurrence of T2DM in the Han population, while a significant increase in Proteobacteria was related to the occurrence of T2DM in the Dai population.

Key Words: Gut microbiota; Type 2 diabetes mellitus; Dai population; Han population; Genetics; Ethnic

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Core Tip: This study revealed that gut microbiota in the Han population is significantly different from the Dai population in healthy children, healthy adults, and patients with type 2 diabetes mellitus (T2DM). There was a significant difference in gut microbiota between healthy children and healthy adults in the Han population, but the difference was less in the Dai population. A significant increase in Bacteroidetes was observed in T2DM patients in the Han population, while a significant increase in Proteobacteria was observed in T2DM patients in the Dai population when compared to healthy controls.

INTRODUCTION

As the world population and the proportion of elderly people increases, type 2 diabetes mellitus (T2DM), a prevalent metabolic disorder, has become a major global public health problem[1]. It is clinically characterized by hyperinsulinemia, insulin resistance, and islet cell damage, which can reach 50% at the time of diagnosis[2]. Individuals with T2DM are highly susceptible to vascular and neurological consequences in addition to life, psychological, and financial stress[3]. In 2021, 537 million people were diagnosed with diabetes, which is expected to increase to 643 million by 2030 and to 783 million by 2045[4]. Although the etiology and pathogenesis of T2DM are still unclear, recent studies have shown that gut microbiota may play key roles[5-8]. Identifying the features of gut microbiota associated with T2DM could help to better understand the pathogenesis of T2DM and prevent or delay the onset of the disease.

The gut microbiota is a complex microecological community composed of more than 100 trillion microorganisms[9-11]. However, it can be affected by various factors, such as age, diet, illness, environment, and genetics[12-14]. Different ethnic groups have a wide variety of dietary patterns, lifestyles, and geographical environments, which can lead to different presentations of gut microbiota and T2DM[15]. Previous studies revealed that there are discriminating microbiota compositions between the Dai and Han populations[16-18]. However, due to the heterogeneity among different ethnic groups, the results of these studies are difficult to replicate. Therefore, it is necessary to carry out analyses of gut microbiota in various ethnic groups with different genetic backgrounds in China.

The Dai ethnic group is a unique minority in Yunnan, China. They have lived in the valley area for generations and generally have endogamous marriages[19]. Due to the different genetic backgrounds, unique lifestyles, and geographical environments[19-22] of the Dai and Han populations, we hypothesized that there may be some underlying differences in the gut microbiota of the two populations. This study was designed to compare the gut microbiota in subjects with and without T2DM in the Dai and Han populations in Yunnan, China. The results of this investigation will help elucidate the

underlying differences of gut microbiota between the Dai and Han populations and determine the association between gut microbiota and T2DM prevalence.

MATERIALS AND METHODS

Subjects recruitment

Healthy children, adult T2DM patients, and ethnically matched healthy adults from the Han and Dai populations living in the same area were recruited and enrolled in this study. Patients with T2DM met the following diagnostic criteria[23]: (1) Fasting blood glucose ≥ 7.0 mmol/L; or (2) Hemoglobin A1c ≥ 6.5%. The enrolled T2DM patients were newly diagnosed and drug-naïve. Subjects who had been treated with antibiotics in the previous 3 mo, were pregnant or lactating, or had inflammatory bowel disease were excluded from the study. The T2DM patients and healthy adults in each population were age-matched ($P > 0.05$). The enrolled subjects have completed the Study Questionnaires to provide personal details and information about health, diet, smoking activity and lifestyle at the time of sample collection. The study was approved by the ethics committee of the Sixth Affiliated Hospital of Kunming Medical University (approval no. 2023-kmykdx6f-66). All participants provided written informed consent.

Sample collection

Fasting venous blood samples were collected from all participants in the morning. After centrifugation (3000 rpm) at 4 °C for 10 min, the serum was immediately extracted and aliquoted. All blood and serum samples were stored at -80 °C until further biochemical analysis was performed. Stool samples were collected into 5-mL disposable sterile tubes within 30 min of discharge and kept at -80 °C until further processing was conducted.

Genomic DNA extraction and 16S rRNA gene sequencing

Total genomic DNA samples were extracted using the OMEGA Soil DNA Kit (M5635-02) (Omega Bio-Tek, Norcross, GA, United States) according to the manufacturer's instructions. The quantity and quality of extracted DNA were measured using a

NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis, respectively. Prepared DNA samples were stored at -20 °C.

The V3-V4 region of 16S rRNA was amplified by polymerase chain reaction (PCR) with the primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The following thermal cycling conditions were utilized: initial denaturation at 98 °C for 5 min; 25 cycles consisting of denaturation at 98 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 45 s; and a final extension of 5 min at 72 °C. PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, United States). After the individual quantification step, amplicons were pooled in equal amounts, and pair-end 2 × 250 bp sequencing was performed using the Illlumina NovaSeq platform with NovaSeq 6000 SP Reagent Kit (500 cycles) at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China).

Sequence and bioinformatics analyses

Microbiome bioinformatics were performed with QIIME2 2019.4 with slight modifications according to the official tutorials (https://docs.qiime2.org/2019.4/tutorials/). Briefly, raw sequence data were demultiplexed using the demux plugin followed by primer cutting with cutadapt plugin. Sequences were then quality filtered, denoised, merged, and chimera removed using the DADA2 plugin. Non-singleton amplicon sequence variants were aligned with mafft and used to construct a phylogeny with fasttree2. Taxonomy was assigned to amplicon sequence variants using the classify-sklearn naïve Bayes taxonomy classifier in feature-classifier plugin. Microbiota comparisons were performed using principal coordinate analysis (PCoA), principal component analysis (PCA), and permutational multivariate analysis of variance (PERMANOVA), diversity index estimate and composition of microbiome analysis.

Biochemical data analysis

Data were analyzed with SPSS Software (Version 29.0; IBM Corp., Armonk, NY, United States). Continuous data were expressed as mean ± SD. Comparisons between groups were performed using one-way analysis of variance and Student's *t* test. Cases with missing data for analysis were omitted, and the remaining data were analyzed. $P < 0.05$ was considered statistically significant.

RESULTS

Subject characteristics

A total of 35 subjects from the Han population (including 15 healthy children, 8 adult healthy controls, and 12 T2DM patients) and 32 subjects from the Dai population (including 10 healthy children, 10 adult healthy controls, and 12 T2DM patients) were enrolled in this study. The main demographics and blood biochemical indexes of the subjects are presented in Table 1. The levels of total bilirubin, direct bilirubin, indirect bilirubin, total cholesterol, hemoglobin A1c, gamma glutamyl transferase, and hypersensitive C-reactive protein were significantly higher in the T2DM patients in the Han population compared to the T2DM patients in the Dai population. Low density lipoprotein cholesterol and apolipoprotein A1 were significantly lower in T2DM patients in the Han population compared to T2DM patients in the Dai population. The levels of triglycerides, total bilirubin, indirect bilirubin, and gamma glutamyl transferase were significantly higher in healthy adults in the Dai population compared to healthy adults in the Han population.

Overall gut microbiota distribution in the Han and Dai populations

The total amount of data comprised 4505369 reads, with an average of 68736 reads per sample. For the 35 Han individuals, the total amount of data included 2132603 reads, with an average of 60931 reads per sample. For the 32 Dai individuals, the total amount of data included 2472766 reads, with an average of 77274 reads per sample. By

clustering analysis at a 97% similarity, 22890 operational taxonomic units were identified.

Analysis of alpha diversity in gut microbiota

Six indices (Chao1, abundance-based coverage estimator, Good's coverage, Shannon, Simpson, and Pielou) were compared between all the population groups to evaluate the alpha diversity in gut microbiota. No significant difference in alpha diversity was observed between healthy children and adults. However, the diversity of the gut microbiota was decreased in T2DM patients compared to healthy adults in both the Dai and Han populations (Figure 1).

Comparison of gut microbiota between healthy children and healthy adults in the Han and Dai populations

PCA scores plot showed that there's differences among groups (Supplementary Figure 1). Further PCoA and PERMANOVA ($P < 0.05$) showed a clear distinction in the gut microbiota between healthy children and healthy adults in the Han population (Figure 2A). The gut microbiota composition of healthy children and healthy adults in the Han population was compared by the Linear discriminant analysis Effect Size (LEfSe) analysis (Figure 2B). The Bacteroidetes phylum was more abundant in the children of the Han population. In the Dai population, there was no clear distinction in the gut microbiota between healthy children and healthy adults after the PCoA (Figure 2C) and the LEfSe analysis (Figure 2D).

These data indicated that the difference in the gut microbiota between healthy children and healthy adults was greater in the Han population than in the Dai population and suggest that the influence of age, diet, or lifestyle on the gut microbiota is greater in the Han population than the Dai population.

Comparison of gut microbiota between healthy adults and adult T2DM patients in the Han and Dai populations

An obvious distinction between healthy adults and T2DM patients in the Han population was observed on the PCoA plot (Figure 3A). The gut microbiota composition of healthy adults and T2DM patients in the Han population was presented in a cladogram (Figure 3B). Compared with healthy adults, the T2DM patients from the Han population had an increased abundance of Bacteroidetes, Bacteroidales, Megamonas, and Bacteroidia within the *Bacteroidetes phylum* (Table 2).

There were no observable differences in the gut microbiota between healthy adults and T2DM patients in the Dai population after the PCoA (Figure 3C) and LEfSe analysis (Figure 3D). Compared with healthy adults, the T2DM patients from the Dai population had an increased abundance of Deltaproteobacteria, Shigella, and Acinetobacter within the *Proteobacteria phylum* (Table 3).

There were several bacterial types with a decreased abundance in T2DM patients in the Han population (63 total types) and in the Dai population (40 total types). Five of these bacteria, including Dorea, Peptostreptococcaceae, Blautia, Ruminococcus, and Coprococcus, were decreased in both populations (Figure 4).

Differences of gut microbiota between the Han and Dai populations during the transition from healthy children to healthy adults and T2DM

To investigate the differences of gut microbiota between the Han and Dai populations during the transition from healthy children to healthy adults and T2DM, the LEfSe analysis was performed (Figures 5-7). Compared to the Dai population, the abundances of *Coprococcus*, *Streptococcus*, and *Lactococcus* within the phylum Firmicutes were consistently higher in the Han population. We also observed that the abundances of Rhizobiales within the phylum Proteobacteria and Veillonellaceae within the phylum Firmicutes were significantly lower in the Han population (Table 4).

DISCUSSION

The relationship between gut microbiota and T2DM is becoming increasingly important. In the past decade, studies have supported the role of gut microbiota in the

pathogenesis of T2DM[24-28]. Some researchers have reported that there are discriminating microbiota compositions between the Han and the Tibetans populations[16-18] and also different among the different ethnicities: Han, Zang, Bai, Hani, Dai, and Miao (including both healthy urban and rural residents of each ethnicity)[29]. However, the underlying differences of the gut microbiota between the Han and Dai populations have not been elucidated. Here, we performed a comparative analysis of the gut microbiota in subjects with and without T2DM from the Dai and Han populations in Yunnan Province, China. To the best of our knowledge, this is the first time to compare the T2DM-associated gut microbiota between Han and Dai populations.

The alpha diversity of the gut microbiota, which reflects the abundance, evenness, and richness[30], might vary between ethnic groups in part due to the varied prevalence of T2DM among ethnic groups[31]. Interestingly, our study showed that there was no significant difference in alpha diversity between the Han and Dai populations, suggesting that the abundance, evenness, and richness of the gut microbiota were not significantly different between the Han and Dai populations. However, the diversity of gut microbiota was decreased in T2DM patients compared to healthy adults in both the Han and Dai populations (Figure 1), which is consistent with the previous results in different populations of the world, including other populations in China[32-34].

The gut microbiota is associated with the age of host[35]. Despite being matched for age (significance less than 0.05), there was a large deviation from the mean value of the age of healthy adults with diabetes in Dai who were actually enrolled in the analysis. We also selected some of these samples with matched age means for subset analysis, and the results were consistent with the existing result. To determine the influence of age on gut microbiota, we also conducted a comparison of gut microbiota between healthy children and healthy adults in the Han and Dai populations. These findings suggested that the difference in the gut microbiota between healthy children and healthy adults was greater in the Han population than the Dai population. The

observed higher relative abundances of genus *Bacteroides* in children and higher relative abundances of genus *Blautia* in adults were consistent with the previous studies[36].

Many researchers have reported that the gut microbiota diversity is affected by T2DM[26,37]. After comparing the gut microbiota between healthy adults and T2DM patients in the Han population, we observed a significant difference in the gut microbiota. However, there was no clear distinction between healthy adults and T2DM patients in the Dai population. The underlying reason behind this warrant further investigation. Moreover, our data showed that the T2DM patients of the Dai population possessed a distinctive microbiota composition characterized by a high abundance of Proteobacteria, which is consistent with the previous results[38]. Recent evidence has also shown that Proteobacteria in gut microbial dysbiosis is essential for metabolic disorders[39]. Interestingly, we found that T2DM patients from the Han population had an increase in Bacteroidetes, Bacteroidales, Megamonas and Bacteroidia within the phylum Bacteroidetes. This discovery conflicted with the results of some earlier studies[32,40]. The possible reason might be that the proportion of Bacteroidales abundance can be altered by high-calorie diets[41], which is also a possible cause of T2DM.

Although we have identified that both age and T2DM influence the gut microbiota, it is unknown which has a greater effect. We explored the differences in bacteria between healthy children to healthy adults and healthy adults to T2DM in both ethnic groups. The results showed that the differences of healthy children between the Han and Dai population were still significant in healthy adults. However, these changes in T2DM patients were not statistically significant. These results demonstrated that the differences were influenced more by age than T2DM during the transition from healthy children to healthy adults and T2DM patients in both the Han and Dai populations.

Several limitations of this study should be taken into account. First, the sample size was relatively small, which limits the generalizability of the findings. It should be confirmed in a larger scale of samples in the future. Second, the effect of gender, food, and smoking activity were not investigated in the study. Third, the metabolic profile

requires further investigation to confirm the relationship between the imbalance of metabolism and gut microbiota alterations.

CONCLUSION

Through the comparative analysis, this study found significant differences in the gut microbiota in the Han and Dai populations, and these differences were influenced to a greater degree by age than by T2DM. Our findings may provide additional insight for further study of gut microbiota dysbiosis-related diseases in the Han and Dai populations.

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