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The association of type II diabetes with gut microbiota composition

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ABSTRACT

It is known that type 2 diabetes (T2D) in humans could be linked to the composition of gut microbiota. The aim of this study was to evaluate three faecal bacterial species, including *Bacteroides fragilis*, *Bifidobacterium longum* and *Faecalibacterium prausnitzii* in patients with T2D. This case control study included 18 patients with T2D and 18 matched persons without diabetes. The concentrations of *B. fragilis*, *B. longum* and *F. prausnitzii* were determined by quantitative Real-Time PCR. Quantitative PCR analysis revealed that the gut bacterial composition in patients with T2D was partially different from that in the healthy individuals. *Faecalibacterium prausnitzii* was significantly lower in patients with T2D (*P*-value = 0.038). *Bacteroides fragilis* was under-represented in the microbiota of the group with diabetes, but its difference between two groups was not significant (*P*-value = 0.38). No difference was observed for *B. longum* community between the both groups (*P*-value = 0.99). Characterization of specific species of intestinal microbiota shows some compositional changes in patients with T2D. The results may be valuable for developing strategies to control type 2 diabetes by modifying the intestinal microbiota. Long-term studies with emphasis on other bacterial groups are suggested to clarify the association of T2D with gut microbiota.

1. Introduction

Type 2 diabetes (T2D) is a metabolic disorder, which is complex and extensive [1]. T2D is caused by insufficient production of insulin from pancreatic beta cells or insulin resistance (inability of body cells to use insulin, properly) [2]. The disease is influenced by both genetic and environmental components. The rapid rise of its incidence in recent decades is considered to be a major public health problem. It has been postulated that intestinal flora genetic factors may be involved in the development of this disease [3]. The gut microbiota is part of a complex network which coordinates the physical and chemical elements of the intestinal barrier together with the immune, sensory, neurological and enteroendocrine systems [4,5]. Creating a stable ecosystem in gut microbiota may protect the host against pathogens [6]. This effect could be attributed to many agents including intestinal pH, microbial interactions, microbial physiological factors, bile acids, drug therapy and immune responses [7,8]. Dysbiosis in communities of gut bacteria can consequence in many chronic diseases such as type 1 diabetes (T1D), inflammatory bowel disease (IBD), obesity, rheumatoid arthritis, allergies, autism and cancer [9]. The human gut microbial metabolic profiles reflect collaboration among the various phylotype, containing more than a thousand species of which about 95% belongs to phylum *Firmicuts* and *Bacteroidetes* followed by phylum *Actinobacteria* and *Proteobacteria* [10–12]. It was suggested that the intestinal microbiome in obese individuals can alter the permeability of intestinal lining and increase the secretion of metabolic endotoxin. This may lead to chronic low level inflammation, impaired insulin resistance and T2D [13]. This low level inflammation can act as trigger in development of diabetes [14,15]. It was demonstrated that composition of gut microbiota may be directly

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responsible for inducing a low-grade inflammatory state closely associated with T2D disorder [16]. It is confirmed that microbiota plays a significant role in development of pre-diabetes conditions such as resistance to insulin [17]. Among intestinal bacteria, the prevalence of *Firmicuts* was significantly lower in patients with type 2 diabetes than those in healthy controls. The *Bacteroidetes*/*Firmicuts* ratio was partially associated to decreased glucose tolerance [16]. It has been indicated that Gram-negative bacteria specifically those belonging to the phylum *Bacteroidetes* and *Proteobacteria* possessed lipopolysaccharide (LPS) endotoxin were relatively enriched in patients with T2D [18]. Furthermore, metabolic endotoxemia (elevated plasma LPS) is significantly related to oxidative stress, macrophage secreted elements and setup inflammatory markers for inducing insulin resistance [19–21]. The degree of microbial diversity may not have a direct link with either health or diabetes, which means it is possible that diabetes is associated with a change in the balance of gut microbiota rather than the action of a single microbe or a simple enhancement in diversity [22]. To our knowledge, there has been no published literature so far to assess *B. fragilis* in T2D patients in comparison with healthy individuals. In addition, the importance of *B. longum* and *F. prausnitzi* in gut flora and its association to some diseases such as IBD, rheumatoid arthritis, T1D and obesity have been highlighted in previous studies [8,18,23,24]. Accordingly, this study was designed to investigate the relationship between these specific bacteria and T2D by comparing the changes in gut bacterial compositions in patients with type 2 diabetes and healthy individuals. The correlations between the studied bacterial species and BMI were also assessed in all study participants.

2. Materials and methods

2.1. Participants and sample collection

Faecal samples were collected from 18 patients with type 2 diabetes (aged 56 ± 8 years) who referred to Institute of Endocrinology and Metabolism Research and Training Center, Iran University of medical Sciences in Tehran, Iran (during March 2015 to February 2016). Specimens were also collected from 18 individuals without diabetes, matched for age, gender and living environment. Inclusion criteria for T2D patients were duration of diagnosed type 2 diabetes less than 5 years and Hemoglobin A1c (HbA1c < 10%).

To ensure comparable data, patients were interviewed for their history of gastrointestinal diseases, and the use of probiotics or prebiotics. Lifestyle habits were assessed in combination with a validated Food Frequency Questionnaire (FFQ) [25], established specifically for Iranian adults. Participants' physical activity levels were also evaluated by International Physical Activity Questionnaire (IPAQ-Short) in both case and control groups [26]. No subject (in both case and control groups) had taken antibiotic, probiotic or prebiotic products for 2 months prior to the study. Fecal samples were collected at three occasions; with a time interval of 2 weeks between the first 2 time points and 10 days between the second and third time points. The samples of the control group were taken at similar time points.

2.2. Stool sample processing and extraction of DNA

Stool samples were stored at −70 °C upon arrival in microbiology laboratory. For DNA extraction, 200 mg of each stool sample was weighed. Total bacterial DNA was extracted from all fecal samples using the QIAamp® DNA Stool mini kit (Qiagen GmbH, Germany) according to the manufacturer's instruction. DNA concentration and quality was determined by Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and agarose gel-electrophoresis. Extracted DNAs were immediately stored at −20 °C.

2.3. Primer and probe designing

Primers and TaqMan probes used in the present study were designed for the first time. These primers and probes target the bacterial 16S rDNA gene. NCBI, www.arb-silva.de, www.probebase.net, www.idtdna.com/calc/analyzer, www.ebi.ac.uk/Tools/msa/kalign/websites and AlleleID (version 7.5) software were used in several stages for designing specific primer and probe sequences. All sequences were aligned by AlleleID software, blast.ncbi.nlm.nih.gov and www.ebi.ac.uk websites. The specific sequences of primers and TaqMan probes were shown in Table 1.

2.4. Real-time qPCR

Bacterial community differences of the three species were measured by real-time TaqMan qPCR in Rotor-Gene 6000 real-time PCR cycler (Qiagen Corbett, Hilden, Germany) using 16S rDNA group specific primers and probes.

All qPCR tests were carried out in duplicate, and averaged numbers were used for calculation. Each reaction mixture with a total volume of 20 μl was composed of 0.5 μl of each primer, 0.5 μl of TaqMan probe, 12 μl TaqMan qPCR Master Mix (Takara. Bio, Shiga, Japan), 1 μl of sample DNA and 5.5 μl sterilized ultra-pure water. Real Time PCR was performed by the following cycle conditions: an initial holding at 95 °C for 30s, followed by 40 cycles of denaturation at 95 °C for 5s, annealing/elongation at 60 °C for 30s, and then a final elongation step at 72 °C for 30s. Negative controls included all elements of the reaction mixture except template DNA and were performed in every analysis. No amplified DNA product was ever detected. The presented data are the mean values of duplicate real-time qPCR analysis.

2.5. Statistical analysis

Results were expressed as mean value ± standard deviation. Statistical analysis was performed with SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA) and Minitab version 16.2.0. Bacterial composition values were converted into fold change (Fold change $= 2^{-\Delta\Delta Ct}$) values before the statistical analysis. To compare two unpaired groups of interval values, the parametric two-sample *t*-test was used. .Correlation between the variables was computed by Pearson correlation. *P*-value <0.05 were considered as statistically significant. The qPCR results were graphically presented by Box and Whisker charts (Minitab 16.2.0).

3. Results

3.1. Subjects

Eighteen Subjects with type 2 diabetes (aged 54.3 ± 7.63 years, BMI: 26.3 ± 3.12 kg/m^2) and 18 healthy subjects (aged 52.1 ± 7.56 years, BMI: 24.43 ± 2.98 kg/m^2) as control were selected in the current study. The group with type 2 diabetes had elevated concentration of plasma glucose as determined by a fasting blood sugar (FBS = 125.1 ± 29.58 mg/dl), glycol hemoglobin (HbA1c = 6.68 ± 1.03%) and 2-h post-prandial blood sugar (2hpp >140). In the control group, FBS and HbA1c were 79.5 ± 6.37 and 4.96 ± 0.54, respectively.

3.2. FFQ analysis

As presented in Table 2, participants in case and control groups