



STUDY ON THE DISTRIBUTIVE CHARACTERISTICS OF INTESTINAL FLORA IN GESTATIONAL DIABETES MELLITUS PATIENTS BY USING HIGH-THROUGHPUT SEQUENCING

Gynaecology

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ABSTRACT

OBJECTIVE: To study the characteristics of intestinal microflora in patients with GDM, and to analyze the diversity and abundance of intestinal microflora. To explore the relationship between GDM and the changes of intestinal microflora.

METHODS: Mid-term pregnant women of 24-28 weeks of gestation in the outpatient department of the Third Affiliated Hospital Zhengzhou University for glucose tolerance test (OGTT) were enrolled in the study. 13 consecutive patients who met the diagnostic criteria for GDM were the experimental group (GH group), 11 consecutive normal pregnant women who matched with age and excluded pregnancy complications were used as a control group (NH group), and collect fecal specimens. High-throughput sequencing of V4 region of bacterial 16S rRNA gene was performed using the Illumina MiSeq sequencing platform, and the results were analyzed by bioinformatics. Using 16S rRNA high-throughput sequencing technology to analyze the difference of intestinal microflora between GDM patients and healthy pregnant women, the results showed that the abundance of intestinal microflora in GDM patients was lower than that in healthy pregnant women, and the distribution of microflora is different. In the GDM group, the intestinal microflora structure was significantly different ($P < 0.05$). At the level of genera, the abundance of *Aggregatibacter*, *Campylobacter*, *Desulfovibrio*, *Gemmiger*, *Leptotrichia*, *Neisseria*, *Odoribacter*, *Oxalobacter*, *Porphyromonas*, *WAL_1855D* were lower than that of healthy control group ($P < 0.05$). Among them, the abundance of *Desulfovibrio*, *Gemmiger* and *Odoribacter* was significantly reduced in GDM patients. LEfSe analysis indicated that methanobacteria, Archaea and *Vibrio* played an important role in the healthy group, while *Haemophilus* and *Pasteurella* played an important role in the gestational diabetes mellitus group.

CONCLUSIONS: The abundance of intestinal microbiota during mid-pregnancy was decreased in GDM patients compared with healthy pregnant women, and the distribution of microflora among individuals varied greatly.

KEYWORDS

Gestational Diabetes Mellitus, Intestinal Microbiota, Species Abundance, Microflora Structure

INTRODUCTION

Gestational diabetes mellitus (GDM) refers to the abnormal glucose metabolism that is first discovered or diagnosed during pregnancy, and has not reached overt diabetes diagnostic criteria. The prevalence of GDM is increasing year by year, it's on the uptrend according to the new diagnostic standard, the prevalence exceeds 15% in global, while more than 10% in China^[1]. GDM not only significantly increases the incidence of macrosomia and Large for Gestational age (LGA)-resulting in dystocia, birth trauma, but also increases the risk of perinatal mortality and Neonatal hypoglycemia, brings to other adverse effects on maternal and fetal health.

Studies in recent years have shown that tens of thousands of bacteria live in the gut of the human body, far more than the number of cells in the body itself. These bacteria make up the body's largest microbial, called gut flora^[2]. Intestinal flora is the main internal environment member of the human body and has been proved to be one of the environmental factors. The new study found that there has a correlation between the occurrence of GDM and the disorder of intestinal

microbial structure, interaction and restriction^[3]. In a healthy human body, undoubtedly, intestinal flora plays a significant role in the normal process of intestine digestion, absorption and energy metabolism, but the imbalance of intestinal microbial community structure may have a certain impact on the behavior and cognitive function of the host^[4]. Therefore, it is very important to explore the changes of the structure characteristics of intestinal flora in GDM patients, and then to screen out the intestinal microorganisms which have clinical value to GDM. In this study, the intestinal flora of GDM patients and normal pregnant women were sequenced by high-throughput sequencing technique, and the differences of species composition between GDM group and normal group were analyzed by bioinformatics technique, to reveal the changes of intestinal flora structure in GDM patients.

MATERIALS AND METHODS

STUDY DESIGN, SETTINGS AND PARTICIPANTS:

It was a hospital based prospective clinical study conducted over a period of eighteen months of eighteen months in the third affiliated hospital in Zhengzhou: 13 Consecutive pregnant women who met the

diagnostic criteria of GDM were the experimental group (GH group) and 11 consecutive normal pregnant women without pregnancy complications (e.g. diabetes with pregnancy, hypertension, obesity, thyroid dysfunction, and intrahepatic cholestasis of pregnancy) were used as a control group (NH group). Subjects who had taken antibiotics and live microorganismal agent within 1 months before sampling; had diarrhea or other gastrointestinal diseases within the past 4 weeks or sample were insufficient or polluted in the collection process were excluded from the study.

METHODOLOGY:

The study was carried out at the Obstetrics & Gynaecology department of third affiliated hospital of Zhengzhou university, China. First, the purpose of the study was explained to the study subjects attending the OPD or Labor room in the local language with the help of the information sheet.

After informed consent had been obtained, a thorough history was taken. Considering inclusion and exclusion criteria, a detailed general, systemic and obstetric examination was carried out. Data regarding age, proteinuria, rapid weight gain, gravida, family history, personal history, other diseases history, dietary habit, BMI, history of antibiotic use, previous obstetric history, oedema, 24 hrs albumins, fetal conditions etc were collected.

Early morning stool sample (2-5 g) was collected in a sterilized sample box and then transferred to -80°C temp within 0.5 hours. then send to lab for 16s rDNA sequencing by PCR methods.

Blood sample was collected in a sterilized test tube, immediately centrifuged the blood and serum was transferred to -80 c temp, then sent to the lab for test.

DATE ANALYSIS

Bioinformatics analysis of high-quality sequences^[5-7]: (1) operational taxonomic units (OTU) cluster analysis: extracted non-repetitive sequences, performed a bioinformatics analysis of the sequences by using the QIIME analysis platform, and Silva database The 16S ribosomal sequence database has been compared, and the sequences with similarity above 97% are merged to generate the classification operation unit OUT; (2) the dilution curve analysis: The R project for statistical computing and graphics is 97%. The similarity level is used to draw the dilution curve of the specimen, and the sample species with different sequencing numbers are compared and the sampling size of the sample is reasonable. (3) The taxonomic analysis of the flora: The R software is used to compare all the sequences in the OTU with the Silva database. Find out the species information that is closest to each other and has a credibility of more than 80%. All sequences in each OTU are analogized to find the species information of the nearest ancestors of different sequences in the same OTU. According to the reference sequence in the Silva library, the OTU was identified. (4) Analysis of the community structure of the flora: According to the results of the taxonomic analysis, the species structure and abundance of the community structure in the sample were analyzed at the genus level.

STATISTICAL ANALYSIS

Data were analyzed and statistically evaluated using SPSS software, version 17 (Chicago II, USA). Quantitative data was expressed in mean, standard deviation while qualitative data were expressed in percentage. Statistical differences between the proportions were tested by chi square test or Fisher's exact test. 'p' value less than 0.05 was considered statistically significant.

ETHICAL ISSUES

All participants were explained about the purpose of the study. Confidentiality was assured to them along with informed written consent. The study was approved by the Institutional Ethical Committee.

OBSERVATIONS & RESULTS

As shown in table 1, Baseline data was comparable between both groups. In GH group, the levels of weight, pre-pregnancy BMI, fasting blood-glucose, OGTT 1h blood-glucose, OGTT 2h blood-glucose, homa insulin-resistance, glycosylated hemoglobin and triglycerides was significantly higher than those in NH group,

Table 1. The subject baseline data

	GH group (n=13)	NH group (n=11)	P
Age(year)	31.20±5.978	29.36±4.007	0.414
Pre-pregnancy BMI(Kg/m ²)	24.7±2.85	21.309±1.553	0.008
FBG(mmol/L)	5.211±0.437	4.555±0.35	0.002
1h blood-glucose (mmol/L)	9.722±1.799	6.845±1.207	<0.001
2h blood-glucose (mmol/L)	9.233±1.694	7±0.953	0.002
Insulin level	11.778±4	8.627±2.927	0.057
Homa insulin-resistance	2.736±0.941	1.765±0.673	0.015
HbA1c (%)	5.411±0.257	5.009±0.353	0.011
TG (mmol/L)	3.11±1.091	2.115±0.68	0.022
CHOL (mmol/L)	5.6±0.908	5.845±1.023	0.582
HDL (mmol/L)	1.823±0.247	2.185±0.343	0.016
LDL (mmol/L)	3.071±0.639	3.189±0.832	0.731

STATISTICAL ANALYSIS OF SEQUENCING QUALITY AND DIVERSITY OF FECAL FLORA

The feces bacterial 16s rRNA genes was amplified by PCR for high-throughput sequencing. Patchwork Tags had been optimized, then selected the samples with minimum number of Tags in all samples, clustering them into OTUs for species classification under 97% similarity. Each sample's abundance information was counted in every OUT. OTU abundance preliminarily illustrated the species richness of the sample.

Figure 1; showed that the Partial least squares discriminant analysis (PLS-DA) based on OUT abundance of samples between two groups. GH group and NH group were clustered and distinguished well. It is suggested that there was a significant difference between two groups.

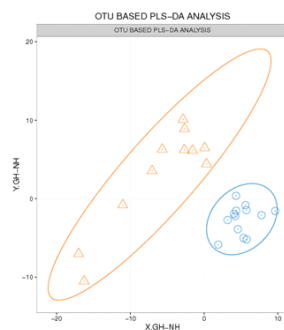


Figure 1. OUT based PLS-DA Analysis of 24 samples

In all 24 samples, the highest abundance was Firmicutes, accounting for 58.5%, followed by Bacteroidetes, accounting for 33%. Proteobacteria and Actinobacteria accounted for 4.5% and 3.0%, respectively; the overall abundance of other bacteria was about 1%.

There was no significant difference in the level of 10 bacterial genera between the two groups (Figure 2); The abundance of Aggregatibacter, Campylobacter, Desulfovibrio, Gemmiger, Leptotrichia, Neisseria, Odoribacter, Oxalobacter, Porphyromonas and WAL_1855D in gestational diabetes mellitus group was lower than that in healthy control group ($P < 0.05$), in which the abundance of Desulfovibrio, Gemmiger and Odoribacter decreased significantly in pregnant women with gestational diabetes.

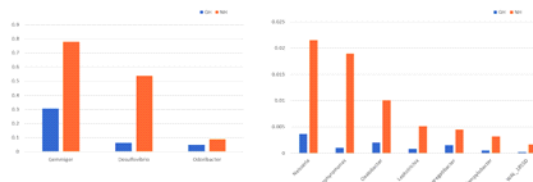


Figure 2. Analysis of the bacteria species with different genera between the two group

DISCUSSION

Intestinal flora is an important part of the human body. Once it is out of balance, it's may cause acute and chronic diseases. Illumina high-throughput sequencing technology is a new sequencing technology^[8],

which applied in the study of intestinal microorganisms at present. The change in Fecal bacteria can reflect the status of intestinal flora. In this study, in order to avoid the influence of host and external factors on the structure of intestinal flora, the gestational diabetes group and healthy control group had similar indicators such as age, pregnancy, diet structure and physical exercise time, and excluded subjects who used antibiotics, prebiotics and probiotics in recent^[9]. In this study, the results showed that the flora abundance of different samples was different, and the variation of the flora in different groups was regular. The 16s rDNA gene is the corresponding DNA sequence responsible for encoding the ribosomal 16s subunit on the bacterial chromosome. There are nine hypervariable regions of V1-V9 in the 16s rDNA gene. Clinically, it is believed that the sequencing of V3-V4 region can well represent all the sequencing results of the 16s region and can be used as a marker for the classification of screening bacteria^[10-11].

In this study, DNA sequencing was carried out on the 16s rRNA-V4 region of fecal samples, and it was indicated that the sequencing amount of the flora was large enough for further biological analysis. The further biological analysis showed that Bacteroidetes and Firmicutes were the dominant flora in pregnant women's intestine, and the analysis result was similar to the results of relevant literature^[13]. But the difference between the two groups was not statistically significant, and the sample size may still need to be further expanded. To some extent, it indicates that the stability of intestinal microbial structure of gestational diabetes mellitus decreases, which is consistent with the previous clinical research results.

The intestinal flora taxonomic analysis of the samples in this study showed that Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria were the main dominant flora, accounting for 58.5%, 33%, 4.5% and 3.0% of the total flora, respectively. The content of other flora was less, accounting for only about 1% of the flora. This discovery consistent with the previous analysis results of human intestinal flora, more than 95% of them can be classified into three phylum: Firmicutes, Bacteroidetes and Proteobacteria, among which Firmicutes and Bacteroidetes account for an absolute advantage^[12]. In order to further analyze the difference, the data of intestinal flora in the two groups were compared at different levels of Phylum, Class, Order, Family and Genus. The statistical analysis at the level of Genus shown that there were significant differences in 10 genera between the two groups. They are *Aggregatibacter*, *Campylobacter*, *Desulfovibrio*, *Gemmiger*, *Leptotrichia*, *Neisseria*, *Odoribacter*, *Oxalobacter*, *Porphyromonas* and *WAL_1855D*. These bacteria genera generally shown a decline in the abundance of pregnant women with gestational diabetes, among which the abundance of *Desulfovibrio*, *Gemmiger* and *Odoribacter* is significantly decreased in pregnant women with gestational diabetes.

A study on the microbiota for obese people have found that the number of Firmicutes in these people was increasing, and the proportions of Firmicutes and Bacteroidetes were changed^[13]. This study suggests that the relative change of gut flora may lead to weight gain and insulin resistance. In this study, the insulin resistance index of GH group was higher than that of NH group. And the difference was statistically significant ($P=0.015$). Intestinal microflora structure analysis of people with different glucose tolerance indicated that most of the microflora were classified into *Blautia*, *Bacteroides*, *Prevotella*, *Clostridium* *Leptum*, and *Clostridium*, and the abundance of *Blautia* in diabetic patients was significantly higher than that in people with pre-diabetes and normal glucose tolerance^[14]. The study found that the species of intestinal flora decreased in third trimester, and the difference of deleted strains among individuals was large, but the number of Actinomycetes and Proteobacteria increased significantly, which was similar to the characteristics of intestinal flora of inflammatory bowel disease and obese people, which might be related to physiological insulin resistance.

The intestinal flora forms a natural barrier on the surface of the intestinal mucosa, participates in digestion and absorption of the human body, and at the same time regulates immune function and prevents the invasion of pathogenic bacteria^[15]. *Clostridium* is closely related to the integrity of the intestinal mucosa, and its structural changes are related to metabolic diseases such as diabetes. It is a research hotspot to reduce the insulin resistance and high blood glucose level of type 2 diabetes by lifestyle intervention and supplementing beneficial bacteria to improve intestinal flora. Similarly, prevention of gestational diabetes by regulating intestinal

flora should also play a role^[16]. As one study shown, the method of adding prebiotics not only reduced the body weight of metabolic syndrome patients, but also reduced the chance of endotoxin-producing pathogens in the intestinal flora, meanwhile, reduced the level of inflammatory indicators in the body, thereby improving insulin resistance^[17]. At present, related microbial modulator drugs have been shown to be safe and effective for the treatment of pre-diabetes and type II diabetes. With the further study on the relationship between gestational diabetes and intestinal flora, it is believed that in the near future it is possible to prevent and treat gestational diabetes by supplementing beneficial bacteria, which is great significance for promoting maternal and child health and reducing the incidence of diabetes.

CONCLUSION & RECOMMENDATIONS

The research on the structure and function of the intestinal flora is just beginning. Which bacteria play the main role in the metabolic diseases such as obesity and type II diabetes, and the mechanism will be revealed by further studies. The results showed that the abundance of gut microbiota in pregnant women with gestational diabetes was lower than that in healthy pregnant women, and the distribution of microflora was different. Among them, in the level of genus, the abundance of *Desulfovibrio*, *Gemmiger*, and *Odoribacter* is significantly reduced in pregnant women with gestational diabetes. It suggested that the intestinal flora may be related to the occurrence and development of gestational diabetes, and the specific mechanism needs further research.

CONFLICT OF INTEREST

The authors have no conflicts of interest.

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