

## Analysis on intestinal flora in patients with non-alcoholic fatty liver disease of different glucose metabolism.

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### Abstract

**Objective:** To analyse the quantitative differences of *Eubacterium rectale*, *Bacteroides thetaiotaomicron*, *Lactobacillus* and *Bifidobacterium* in non-alcoholic fatty liver patients with type 2 diabetes and non-alcoholic fatty liver patients with normal glucose metabolism to explore the relationship of intestinal flora and the occurrence and development of non-alcoholic fatty liver disease and diabetes.

**Methods:** 100 cases of Nonalcoholic Fatty Liver Disease (NAFLD) patients with type 2 diabetes, 50 cases of NAFLD patients with normal glucose metabolism and 60 cases of normal metabolism without NAFLD (normal control group) were selected as research objects. The total DNA of faeces was extracted and the expression level of DNA of target bacteria was detected by relative quantification of Real Time PCR.

**Results:** The quantity of *Eubacterium rectale* and *Lactobacillus* in NAFLD patients with type 2 diabetes group was significantly higher than that of NAFLD patients with normal glucose metabolism group, the difference of which was statistically significant ( $P=0.00$ ,  $P=0.006$ ), and *Bacteroides thetaiotaomicron* decreased significantly, the difference of which was statistically significant ( $P=0.002$ ), and the quantity of *Bifidobacterium* presented no significant difference ( $P>0.05$ ). The quantity of *Eubacterium rectale* and *Lactobacillus* in NAFLD patients with type 2 diabetes group were significantly higher than that of normal control group, the difference of which was statistically significant ( $P=0.00$ ), and *Bacteroides thetaiotaomicron* as well as *Bifidobacterium* decreased significantly, the difference of which was statistically significant ( $P=0.00$ ). The quantity of *Bifidobacterium* in NAFLD patients with normal glucose metabolism group decreased significantly normal compared to normal control group, the difference of which was statistically significant ( $P=0.00$ ), and *Eubacterium rectale*, *Bacteroides thetaiotaomicron* and *Lactobacillus* had no significant difference ( $P>0.05$ ).

**Conclusion:** Intestinal flora in patients with non-alcoholic fatty liver disease of different glucose metabolism changes much.

**Keywords:** Non-alcoholic fatty liver disease, Type 2 diabetes, Intestinal flora, Gut-liver axis.

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### Introduction

#### Prevalence of nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) refers to a clinical pathological syndrome mainly characterized by the diffuse hepatic bullous steatosis and caused by other than alcohol and other liver damage factors, which includes simple hepatic steatosis (NAFL), Nonalcoholic Steatohepatitis (NASH) and the related liver fibrosis, cirrhosis and hepatocellular carcinoma [1]. In recent years, the prevalence rate of NAFLD showed a rising trend. In the western developed countries, prevalence rate of NAFLD in common adults was 20%~33% [2,3]. And in China's developed areas such as Shanghai,

Guangzhou and HK, prevalence rate of NAFLD in adults was around 15% [4].

#### The relationship between NAFLD and type 2 diabetes

Prevalence rate of NAFLD in patients with type 2 diabetes was higher, and a study from Italy showed that 70% of outpatients with type 2 diabetes met the diagnosis criteria of NAFLD [5]. NAFLD also increases the incidence of type 2 diabetes. Japanese scholars found that the annual incidence rate of type 2 diabetes was 1.7%, five years' incidence rate was 6.8%, and 10 y' incidence rate was 17.7% during the following up of NAFLD patients [6]. NAFLD could be a consequence of diabetes, and some scholars believed that NAFLD may be a

pre diabetic stage [7] and considered NAFLD patients as new reserve force of type 2 diabetes.

### ***Influence of intestinal flora on NAFLD and type 2 diabetes***

The relationship between intestinal flora and NAFLD, type 2 diabetes is a hot topic at present. As important environmental factors going into the human body, the intestinal flora affects nutrition, metabolism and immunity of the host, and plays a very important role in diseases of obesity, diabetes, metabolic syndrome and NAFLD, etc. The formation of metabolic endotoxemia [8] from intestinal flora is involved in the occurrence and development of metabolic disease. At present, domestic researches are basically limited to the category of intestinal flora and NAFLD or type 2 diabetes without the research of NAFLD associated with type 2 diabetes. The method of real-time fluorescence quantitative PCR was adopted in this experiment for patients with NAFLD associated with type 2 diabetes and NAFLD associated with normal glucose metabolism. The *Firmicutes* refers to *Eubacteriu rectale*, *bacteroidetes* represents *Bacteroides thetaiotaomicron*, and probiotic represents changes of *Lactobacillus* and *Bifidobacterium*.

### ***Object***

A total of 210 people participated in the study from physical examination center, outpatient department and hospitalization in our hospital from November 2011 to January 2013, aged 21-85 y old. They were divided into 3 groups: (1) 100 patients with NAFLD complicated with type 2 diabetes; (2) 50 cases of NAFLD patients with normal glucose metabolism; (3) 60 cases of normal metabolism of the patients without NAFLD (normal control group). Diagnostic criteria for type 2 diabetes mellitus referred to the literature [9]. The clinical diagnosis of NAFLD is based on the recommended diagnostic criteria from guidelines for the diagnosis and treatment of nonalcoholic fatty liver disease (2010 Edition) [10] made by fatty liver and alcoholic liver disease group of Hepatology branch in Chinese Medical Association, which need comply with the following 3 conditions: (1) no history of smoking or drinking equivalent to alcohol content less than 140 g/week (female < 70 g/week); (2) except the specific diseases which can lead to fatty liver such as viral hepatitis, drug-induced liver disease, total parenteral nutrition, hepatolenticular degeneration, autoimmune liver disease; (3) the histological changes of liver biopsy are consistent with the pathological diagnosis criteria of fatty liver disease. In view of the liver histological diagnosis difficult to obtain, NAFLD is defined as: (1) liver imaging manifestations conform to the diagnosis standard of diffuse fatty liver and no other reason for interpretation; and (or) (2) Alanine Aminotransferase (ALT) and (or) Aspartate Aminotransferase (AST), Glutamyl Endopeptidase (GGT) increased continuously for more than half a year with unexpected reason in patients with metabolic syndrome related components. After weight loss and improved IR, abnormal enzyme spectrum and imaging of fatty liver improved even normal can be diagnosed as

NAFLD. GE LOGIQ9 color ultrasonic diagnostic instrument was used for abdominal ultrasound examination: according to the NAFLD guidelines for Asia Pacific region [11], more than two of the following abnormalities found after abdominal ultrasonography can be diagnosed as hepatic steatosis: 1. near field echo of the liver is enhanced, and the far field echo of liver is weakened; 2. liver parenchyma echo is dense and stronger than the renal parenchyma; 3. the display of blood vessels of liver and structure of the bile ducts is not clear.

## **Materials and Methods**

### ***Collection of specimens***

Stool specimens of about 2 g were collected from the subjects within 4 h and put into a sterile dry bottle, then numbered and immediately placed at -80°C in the refrigerator to save.

### ***Extraction of fecal DNA***

0.25 g fecal samples were weighed and fecal DNA was extracted by Ultra Clean Fecal DNA Isolation Kit (MoBio kit company, USA) and the purity of DNA was tested. Absorbance of the total DNA in 260 nm and 280 nm was detected by UV spectrophotometer to obtain the ratio of concentration of total DNA and OD 260/OD280. If the ratio is between 1.6 and 1.8, it means the sample DNA was of high purity without protein and RNA pollution; if the ratio is less than 1.6, it indicated the protein pollution; if the ratio is between 1.8 and 2, it represents the pollution of RNA [12].

### ***Synthesis of primers***

According to the gene sequence of *Eubacteriu rectale*, *Bacteroides thetaiotaomicron* and *GAPDH* (Reference) in GenBank, specific PCR primers were designed by premier5.0 and primer sequences after design and full sequence in the Blast database were compared and verified, and *Lactobacillus*, *Bifidobacterium* primers sequence could be respectively found in reference [13,14]. All the primer of primer sequence was synthesized by Shanghai Jierui Biotechnology Company. Upstream primer of *Eubacteriu rectale*: 5-GGGTGACCGGCCACATTGGG-3, downstream primer: 5-ATCAGACTTGCCGCACCGCC-3, product 278 BP; upstream primer of *Bacteroides thetaiotaomicron* 5-CCGCTCCGTTAGCTGCGTG-3, downstream primer 5-ACGTAGGCTGCACAGCCGGT-3, product 202 BP; upstream primer of lactic acid bacteria 5-AGCAGTAGGGAATCTTCCA-3, downstream primer 5-CACCGCTACACATGGAG-3, product 341 bp; upstream primer of *Bifidobacterium* 5-GATTCTGGCTCAGGATGAACGC-3, downstream primer 5-CTGATAGGACGCGACCCAT-3, product 230 bp; upstream primer of *GAPDH* 5-TCGGCATCATCGAAGGTCTG-3, downstream primer 5-TGCCATTCAAGTTCTGGCAGT-3, product 171 bp.

### Amplification system

The reaction solution was configured in PCR tube (PCR reaction system 25  $\mu$ l: FastStart Universal SYBR Green Master (Rox) (2X conc) 12.5  $\mu$ l, specific upstream primers of sequence 1  $\mu$ l, specific downstream primers of sequence 1  $\mu$ l, template DNA 1  $\mu$ l, RNase Free Water 9.5  $\mu$ l), and PCR reaction was conducted.

### The detection of target bacteria in samples

Extraction of fecal DNA samples (210 cases) were respectively carried out by real-time fluorescence quantitative PCR reaction of 4 kinds of target bacteria and *Escherichia coli*. The PCR reaction conditions of *Eubacterium rectale*, *Bacteroides thetaiotaomicron*: polymerase activation for 10 min at 95°C; melting cycle for 15 s at 95°C, annealing/extension for 1 min at 65°C with a total of 40 cycles. PCR reaction conditions of *Bifidobacterium*: polymerase activation for 5 min at 95°C; melting cycle for 15 s at 95°C, annealing/extension for 1 min at 60°C; 45 s at 72°C with a total of 35 cycles. PCR reaction conditions of *Lactobacillus*: polymerase activation for 10 min at 95; melting cycle for 15s at 95, annealing / extension for 1min at 61.6with a total of 35 cycles. PCR reaction conditions of GAPDH: polymerase activation for 30s at 95°C; melting cycle for 5 s at 95°C, annealing/extension for 5 s at 61°C, 34 s at 72°C with a total of 40 cycles. The blank control and two random repeat samples were set at the same time in each experiment and after reaction, ABI Prism 7300 fluorescence quantitative PCR instrument automatic drawn amplification power curve melting curve. Ct value was analysed by computer (Ct value refers to amplification cycle times when fluorescence signal of amplification product reached the set threshold in PCR amplification process). Due to Ct value and gene copy quantity in the template presented linear relationship, which meant the value of Ct and expression level of DNA were negatively related, and the higher the Ct value, the lower the expression level of target gene. The  $2^{-Ct}$  method was adopted to calculate the relative expression quantitative of DNA and calculation formula is as the following:

$Ct_{\text{target gene}} = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$  (*GAPDH* as reference gene)

$Ct = Ct_{\text{target gene}} - Ct_{\text{calibration value}}$ , the data in first group as calibration values

### Detection of PCR products

1. Gel electrophoresis: 2% agarose gel was prepared, the PCR products were detected by electrophoresis, the gel imaging was conducted by Tanon 3500 UV image analysis system scanner, fragment band positions of PCR amplification product were compared with DNA ladder Maker to determine fragment size of amplification product with observation of results under UV light as well as photographing and recording. 2. Sequencing verification: PCR products 50  $\mu$ l of the determined target fragment were sent directly to Dalian Bao Biological Engineering Co. Ltd to detect the sequence.

### Statistical processing

Excel 2007 was used to set up a database, and SPSS 17. 0 statistical software was adopted. The expression quantity of four kinds of target bacteria was non-normal distribution of measurement data expressed by the median of M (P25, P75). When the comparison of two groups of samples met the normality and homogeneity of variances, t test of the two sample was used; when not meeting the condition, non-parametric test of rank transformation (Mann-Whitney U test) was adopted;  $P < 0.05$  was taken as standard of the level of significant difference.

## Results

### The detection of DNA purity

It was determined by UV spectrophotometer. If the ratio of total DNA to A260/A280 in fecal samples was between 1.6 and 1.8, it showed that the purity of DNA was high and there was no pollution.

### Analysis of PCR products

Baseline was set according to the amplification curve and Ct value for each sample was calculated, because of Ct value and gene copy quantity of template presenting the logarithmic linear relationship, gene copy quantity of template can be indirectly understood according to the value of Ct.

### The detection of target bacteria and GAPDH amplification products in samples

After the reaction, PCR products amplified by 4 kinds of target bacteria were analysed by 2% agarose gel electrophoresis and Tanon3500 UV image, and a clear band appeared at the corresponding position, and the sizes were basically consistent compared with the Marker, as shown in Figures 1-4.

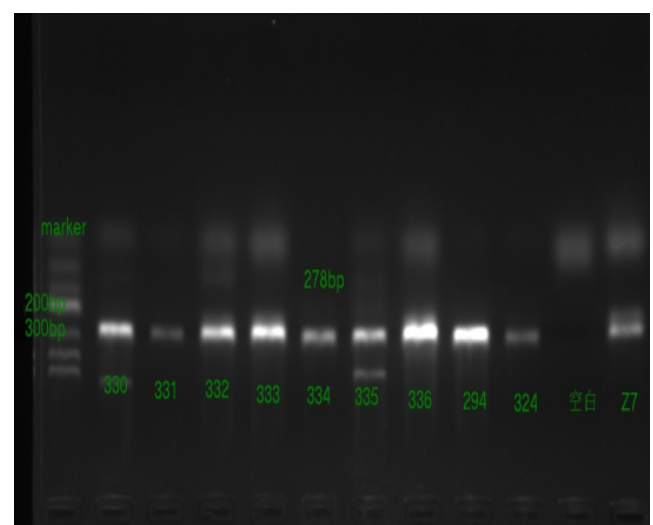


Figure 1. Gel electrophoresis figure of amplification product in *Eubacterium rectale*.

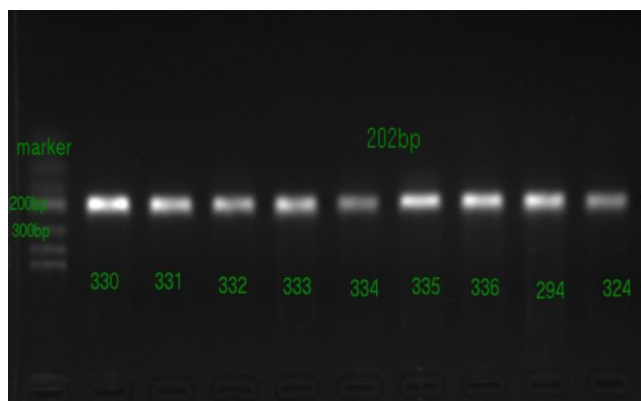


Figure 2. Gel electrophoresis figure of amplification product in *Bacteroides thetaiotaomicron*.



Figure 3. Gel electrophoresis figure of amplification product in *Lactobacillus*.



Figure 4. Gel electrophoresis figure of amplification product in *Bifidobacterium*.

**Sequencing results**

Sequencing results were compared by Blast and Genebank (<http://ncbi.nlm.nih.gov/blast>). Results showed that the amplification product sequences were respectively consistent with those of *Eubacterium rectale*, *Bacteroides thetaiotaomicron*, *Lactobacillus*, *Bifidobacterium*.

The comparison of target bacteria in intestinal tract in NAFLD patients with type 2 diabetes group and NAFLD patients with normal glucose metabolism group and normal population.

According to glucose metabolism situation, the NAFLD patients were divided into NAFLD with type 2 diabetes group 100 cases (A group) and NAFLD patients with normal glucose metabolism group 50 cases (B group), comparing respectively to 60 cases of normal control group (C group). The intestinal target bacteria expression quantity of three groups of subjects

is shown in Table 1. And statistical results of the comparison are shown in Table 2. The results are as follows.

**1. NAFLD patients with type 2 diabetes group and NAFLD patients with normal glucose metabolism group:** *Eubacteriu rectale* quantity in NAFLD patients with type 2 diabetes group was significantly higher than that of NAFLD patients with normal glucose metabolism group, the difference of which was statistically significant (P=0.00), and the quantity of *Lactobacillus*, *Bacteroides thetaiotaomicron* decreased significantly, the difference of which was statistically significant (P=0.006, P=0.002), and the quantity of *Bifidobacterium* presented no significant difference (P=0.175).

**2. NAFLD patients with type 2 diabetes group and normal control group:** *Eubacteriu rectale* and *Lactobacillus* quantity in NAFLD patients with type 2 diabetes group were significantly higher than that of normal control group, the difference of which was statistically significant (P=0.00, P=0.00), and *Bacteroides thetaiotaomicron* and *Bifidobacterium* decreased significantly, the difference of which was statistically significant (P=0.00, P=0.00).

**3. NAFLD patients with normal glucose metabolism group and normal control group:** *Eubacteriu rectale*, *Bacteroides thetaiotaomicron* and *Lactobacillus* and quantity in NAFLD patients with normal glucose metabolism group had no significant difference compared to that of normal control group. *Bifidobacterium* decreased significantly, the difference of which was statistically significant (P=0.00).

Table 1. The relative expression level of target bacteria of subjects of this three groups; Expressed as: (25 Percentile, 75 Percentile).

	<i>Eubacteriu rectale</i>	<i>Bacteroides multiforme</i>	<i>Lactobacillus</i>	<i>Bifidobacterium</i>
Group A	0.790, 3.092	0.641, 5.117	0.002, 0.389	0.257, 2.814
Group B	0.142, 0.436	0.985, 11.256	0.004, 0.105	0.222, 1.165
Group C	0.003, 0.340	1.853, 6.267	0.001, 0.023	1.052, 10.601

Table 2. The quantity of target bacteria comparison of the three groups.

	Groups A and B		Groups A and C		Groups B and C	
	Statistic	P	Statistic	P	Statistic	P
<i>Eubacteriu rectale</i>	-4.28	0	-4.88	0	-0.67	0.505
<i>Bacteroides multiforme</i>	-3.05	0.002	-3.49	0	-0.48	0.631
<i>Lactobacillus</i>	-2.77	0.006	-3.67	0	-0.14	0.885
<i>Bifidobacterium</i>	-1.38	0.175	-4.29	0	-4.79	0

**Discussion**

Certain studies have shown that intestinal microflora structure had no change without endotoxemia on the condition of NAFL. When NAFL progressed to NASH and liver fibrosis, the imbalance of intestinal flora structure, small intestinal bacterial

overgrowth and endotoxemia appeared [15,16]. In our previous study, we found that NAFLD patients had abnormal glucose metabolism [17] (including patients with NAFLD complicated with type 2 diabetes and normal glucose metabolism). Haukeland, etc., [18] studied and found that abnormal glucose metabolism might suggest that NAFLD patients progressed to NASH and hepatic fibrosis. Type 2 diabetes is a strong predictor of NASH and hepatic fibrosis [19]. Then we divided NAFLD group into NAFLD with type 2 diabetes group and NAFLD patients with normal glucose metabolism group, and analysed changes of intestinal bacteria the structure in different status of glucose metabolism in patients with NAFLD.

The "two hit" hypothesis in the pathogenesis of NAFLD pointed out that [20] in the two hit of promoting the liver to progress into NASH, intestinal factors (intestinal flora, mucosal barrier, etc.) played an important role. The imbalance of intestinal microflora, intestinal bacterial overgrowth, increased intestinal permeability and increased endotoxin into the blood, the formation of endotoxemia and endotoxin stimulating release of inflammatory factor from liver Kupffer cells, as well as causing inflammatory reaction, induced the fatty degeneration of liver cell to appear necroinflammation and fibrosis.

*Eubacterium rectale*, polymorphic bacillus, *Lactobacillus*, *Bifidobacterium* play an important role in the host's energy metabolism, immunity, maintaining the stability of intestinal flora and integrity of intestinal flora of intestinal mucosa barrier function. We observed in NAFLD with type 2 diabetes group, the quantity of *Eubacterium rectale*, *Lactobacillus*, *Bacteroides* were significantly different from those of NAFLD with normal glucose metabolism group and normal control group; and there was only the difference of *Bifidobacterium* quantity between NAFLD with normal glucose metabolism group and normal control group, suggesting that when NAFLD combined with abnormal glucose metabolism appeared, probably due to the aggregation of abnormal metabolism factors, changes of intestinal microflora were caused, breaking the intestinal-liver axis balance and promoting the subsequent development of disease. It was speculated that the target bacteria might be involved in the process of NAFLD progressing from normal glucose metabolism to type 2 diabetes, and promoting type 2 diabetes to develop into NASH and liver fibrosis, liver cirrhosis through the two hit. Intestinal flora may play an important role in NAFLD to type 2 diabetes and subsequent progression process of disease.

In conclusion, we found that in the intestinal tract of NAFLD patients with type 2 diabetes, *Eubacterium rectale*, *Lactobacillus*, and *Bacteroides thetaiotaomicron* had changes of the quantity, and only *Bifidobacteria* have obvious difference in NAFLD patients with glucose metabolism, which provided the data for us when studying the relationship of intestinal flora and metabolic diseases.

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