Correlation analysis for the alterations of intestinal flora and their metabolite in hepatocellular carcinoma patients

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Abstract

Hepatocellular Carcinoma (HCC) is one of the most common malignant tumors in the world. There are a variety of treatment options for HCC, such as hepatectomy, radio frequency ablation, radiotherapy and chemotherapy, liver transplantation, etc. However, due to the characteristics of low early diagnosis rate, high malignancy and rapid progression of HCC, the majority of diagnosed patients are in the middle or late stage. Therefore, new research ideas are needed for the prevention, diagnosis and prognosis of HCC.

Accumulating evidence reveals that liver disease patients show different intestinal flora composition compared to healthy people and the change of intestinal flora may promote the carcinogensis of HCC via disturbing immune regulation. Here, we studied the differences in intestinal floras composition and interleukin (IL) indexes between HCC patients and healthy people. A total of 64 HCC patients and 24 healthy people were recruited, and their fresh stool samples and serum samples were collected for 16S rRNA sequencing and metabolite index measurement. Our data showed that 484 operational taxonomic units (OTUs) and 476 OTUs were detected in the HCC and control groups, respectively. Moreover, we found that most liver function indexes, IL-6 and IL-10 expression levels were significantly different between the two groups. Of note, the abundances of Actinomycetes, Faecalibacterium, Lactobacilli and Bacteroides were significantly higher in HCC group than the control group, and the change was associated with increased plasma levels of IL-6, IL-10 and IL-12 in HCC patients. In conclusion, the abundance of intestinal floras in the HCC group was different from the control group. Additionally, in HCC patients, the expression levels of IL-6 and IL-10 were higher than health people, and the difference showed a strong correlation to the abundance of some intestinal floras.

Background

Hepatocellular Carcinoma (HCC) is one of the most malignant tumors in the world and also a severe worldwide health threat\(^1,2\). There were more than 900,000 people suffered from HCC every year, and the morbidity and mortality of HCC ranked the fourth and sixth, respectively, among all tumors\(^3,4\). The risk factors for HCC include genetic factors, hepatitis virus B infection, aflatoxin and alcohol abuse\(^5-7\). Due to its occult occurrence, the majority of HCC patients are already in their advanced stage when they are diagnosed, and their median survival time for advanced liver carcinoma is less than one year\(^8-10\). However, if the HCC patients are treated at early, the rate of 5-year survival can increase to about 70\(^%\)\(^11\). Therefore, early diagnosis and treatment are of critical importance in improving the survival cycle of HCC patients.

Intestinal flora can be regarded as a "self-assembly" organ, with a large amount of bacteria existing in a certain proportion and reaching a certain equilibrium state\(^12\). If the homeostasis of intestinal flora is broken, it may lead to abnormal increase of certain metabolic small molecule products, and then promote the occurrence and development of various diseases\(^13\).
In recent years, many studies have shown that intestinal epithelial dysfunction, abnormal bile acid metabolism resulting from the changes in intestinal flora composition may promote the occurrence and development of HCC\textsuperscript{[14–16]}. Intestinal floras can regulate the bioavailability of glycine, which on the one hand, reduces the antioxidant capacity of small intestine, liver and colon, and on the other hand, disturbs the methylation of DNA histones and the metabolism of protein and purine, thus promoting the proliferation of cancer cells\textsuperscript{[17]}. Intestinal flora may also be involved in inhibiting immune surveillance of hepatocellular carcinoma cells \textsuperscript{[18,19]}. For example, elimination of Gram-positive bacteria from the intestinal tract by vancomycin prevents tumor occurrence, while colonization of Clostridium in the intestinal tract promotes tumor growth\textsuperscript{[20]}. In addition, symbiotic bifidobacteria can enhance anti-tumor immunity and regulate therapeutic effects by blocking programmed cell death ligand 1 (PD-L1) \textsuperscript{[21]}. In recent years, intestinal flora research has attracted great attention for studying the etiology of many diseases\textsuperscript{[22–24]}. The human gastrointestinal tract is one of the most diverse ecosystems, containing approximately $10^{13}$ species of microorganisms\textsuperscript{[25]}. In the intestinal flora of human body, Bacteroidetes and firmicutes occupy the main position, followed by actinobacteria, verrucomicrophyla and proteobacteria, etc., which exist in a certain proportion in the intestinal tract and reach a balance state\textsuperscript{[26]}. Intestinal flora is regarded as a multi-functional "virtual organ", which is important to the production of bioactive metabolites, immune regulation, energy dynamic balance and pathogen protection\textsuperscript{[27–29]}. More and more studies have demonstrated that intestinal flora is involved in a variety of physiological and pathological processes, in which it can not only promote the development of diseases through local effects, such as inflammatory bowel disease\textsuperscript{[30]}, but can also affect the development of other organ-related diseases, such as the nervous system\textsuperscript{[31]}, cardiovascular system\textsuperscript{[32]}, liver\textsuperscript{[33]}, lung\textsuperscript{[34]} and kidney\textsuperscript{[35]}. Most HCC develop from chronic liver disease, and these chronic liver diseases are associated with the alternations of intestinal floras\textsuperscript{[36,37]}. As an example, the changes in the intestinal barrier maybe lead to intestinal leakage, and thus expose the liver to microbial associated molecular pattern (MAMP) and bacterial metabolites, thus promoting the progression of chronic liver diseases\textsuperscript{[38]}. The cell wall of Gram-negative bacteria is composed of lipoprotein, lipid bilayer and lipopolysaccharide (LPS)\textsuperscript{[39]}. The plasma LPS level has been shown to be increased during the development of liver cancer in diethylnitrosamine (DEN)-treated rats, which was decreased after antibiotic treatment\textsuperscript{[40]}. The change of intestinal flora has also been demonstrated to be associated with the formation of liver cancer in DEN-treated mice, which was accompanied by increased abundance of Gram-negative bacteria (Colibacillus, Atopobium, Egertella, etc.), the reduction in contents of some probiotics (such as Lactobacillus, Bifidobacterium and Enterococcus) as well as the increased levels of plasma LPS\textsuperscript{[41]}. Taken together, intestinal flora is closely related to the occurrence and development of HCC, but how intestinal flora affects the immune system is still an open question\textsuperscript{[42–44]}. 
In this study, we applied 16S rRNA sequencing to study the dynamics of the microbiota in HCC patients and to predict the critical bacterial and metabolic functions through bioinformatics analysis. The results of intestinal flora analysis showed that diversity of intestinal flora in patients with HCC was significantly lower than that in healthy people and this change was accompanied by the alterations of several interleukins.

**Methods**

**Recruitment of Volunteers**

We recruited 320 volunteers who diet is relatively identical, from Mar.2019 to Jun.2019 in HUNAN CANCER HOSPITAL and staged their tumor status with Diagnosis and treatment of primary carcinoma of the liver (2017). According to inclusion and exclusion criteria, we excluded 152 patients who had serious cardiovascular or metabolic diseases, 12 patients who had Immune system diseases, 8 patients who had digestive system diseases, 5 patients who had other cancer, 55 patients who had taken antibiotics or probiotics related preparations for less than a month. Finally, we recruited 64 patients who were allocated to Primary carcinoma of the liver group (HCC group), and 24 healthy volunteers to control group. The experiment was approved by the ethics Committee of Longhua District People's Hospital of Shenzhen. The committee's reference number is 2021.054. And all methods were carried out in accordance with relevant guidelines and regulations.

Recruitment standards:

(1) HCC group: patients in compliance with primary liver cancer diagnosis and treatment specifications (2017 edition).

(2) Control group: healthy volunteers matched with age, sex and body mass index (BMI) of HCC group.

Exclusion Criteria:

(1) Serious cardiovascular disease or metabolic disease (heart failure, diabetes, hypertension, etc.).

(2) Autoimmune diseases (systemic lupus erythematosus, rheumatoid arthritis, etc.).

(3) Other diseases of digestive tract system (gastritis, gastric ulcer, inflammatory bowel disease, etc.).

(4) Other tumors.

(5) Mental illness.

(6) Antibiotics and antihypertensive drugs were used in the last month.

(7) Probiotics, prebiotics and other microbial preparations were used in the past one month.
For both HCC group and control group, all patients were yellow race. There were 57 male and 7 female, and the ages were among 41 to 71 (mean ± sd = 55.97 ± 8.03) in HCC group. And there were 21 male and 3 female in control group, and ages were among 41 to 70 (56.75 ± 9.84). The differences on gender (chi-square = 0.04, Fisher’s Exact test p = 0.55) and age (t = 0.38, p = 0.70) between two groups had no statistical significance (both p < 0.05). Mean ± SD of BMI in HCC group was 18.83 ± 1.26, while it in control group was 19.75 ± 1.59. The t test for BMI between two groups showed that t = 2.84 and p = 0.006.

**Sample collection**

**Sample for intestinal Microbes Detection**

Feces from patients were collected to 16S rDNA amplification Sequencing for intestinal microbes. The feces were emptied into the disposable toilet bowl, and about 3g of feces (pay attention to avoid the contaminated part such as urine and blood) were picked up with the picking rod of the sterile feces collector and placed in the sterile collector. Then we ranked the sterile collector and put it into the −80°C storage immediately(within 1 hour).

**Sample for Serological detection**

Blood from vein was drew by nurses followed sterile and standardized operating procedures. And the blood was centrifuged at 4000rpm for 4minutes, then the supernatant was collected and picked into 0.5ml centrifuge tube and stored at -80°C(avoid repeated freeze-thaw cycles).

**Detection in the intestinal flora**

**DNA Extraction**

Total DNA was extracted by GenMagBio kit according to the manufacture’s instructions. The 16S V3-V4 variable region was subjected to PCR amplification using the 338F (5′-ACTCCTACGGGAGGCAGCA G-3′) and 806R(5′-GGACTACHVGGGTWTCTAAT-3′) primers. **16S rDNA Sequencing**

The PCR products were isolated using a 2% agarose gel, purified and quantified. A PE 2∗300 library was constructed using the purified amplification fragments according to the standard procedure of the TruSeqTM DNA Sample Prep Kit (Illumina, San Diego, USA). Finally, the DNAs were sequenced using the Miseq PE300 sequencer (Illumina, San Diego, USA).

**Detection of Blood specimens**

**Liver function test**

Clinical indexes of liver function were detected by automatic biochemical analyzer (Roche, Cobas 8000). Total protein(TP), albumin(ALB), globulin(GLO), total bile acids(TBA), total bilirubin(TBIL), direct bilirubin(DBIL), indirect bilirubin(IBIL), alanine transaminase(ALT) and aspartate transferase(AST) were collected.
IL-6, IL-10 and IL-12

Serum samples for IL-6 were measured by using Up-converting Phosphor Technology. While Serum samples for IL-10 and IL-12 were measured by ELISA method.

Statistical analysis

Test level \( \alpha = 0.05 \). Mean ± standard deviation was used for statistical description of measurement data, and student-T test was used for statistical inference. The median (quartile) was used for statistical description and chi-square test was used for statistical inference.

Results

DNA sequencing results of intestinal flora and statistical analysis

Species abundance and diversity

In this process, regions V3-V4 of 16S rDNA were selected to be sequenced. A total of 4,976,854 available raw readings were obtained from all 88 samples, with an average reading of 56555.16 ± 13201.23 per sample (Fig. 1A). All readings were done by CD-HIT clustering and NAST alignment, as a result, 510 operational taxonomic units (OTUs) were generated from 4,976,854 readings. 484 OTUs were detected in the HCC group, and 476 OTUs were detected in the health group. Further analysis showed that there were 34 OTUs for HCC group only, while 26 OTUs for health group, and 450 OTUs for both groups (Fig. 1B, 1C venn and heatmap). The results of rarefaction curve and species accumulation curve constructed from sequenced data indicated that the sequencing depth was stable, and the diversity of most of the microbiome were included in the samples (Fig. 2A,2B).

The Metastats analysis results showed that the number of colonies with statistically difference (p < 0.05) in abundance in order of phylum, class, order, family, genus and species were 5, 6, 10, 15, 23 and 19. At the phylum level, the abundance of Proteobacteria, cyanobacteria and TM7 in HCC group was higher than that in control group, while the Firmicutes and Tenericutes was decreased (Fig. 3). At species level, the abundance of Dentocariosa, Mucilaginosa, Acidifaciens, Ovatus, Anginosus, Infantis, Neonatale, Obeum, Citroniae, Catus, Eutactus, Satelles, Flavefaciens, Neonatale, Noxia, Dispar, Moorei, D168, and Segnis parainuenzae was signicantly different in two groups (Fig. 4). And the results of LEfSe analysis were shown as Fig. 5A and 5B.

For control group, the Chao1 index was 216.39 ± 46.49(mean ± standard deviation(std)), the ACE index was 217.71 ± 44.36, the Shannon index was 4.71 ± 0.84, the Simpson index was 0.91 ± 0.07. For HCC group, the results of alpha diversity analysis showed the Chao1 index was 175.58 ± 42.66, the ACE index was 175.71 ± 40.31, the Shannon index was 3.96 ± 0.09, the Simpson index was 0.86 ± 0.13. Comparing
the four kinds of index between two groups showed all p-values were less than 0.05, indicated that the diversity of control group was more abundant than HCC group (Table 1 and Fig. 6).

<table>
<thead>
<tr>
<th>Difference analysis of Alpha diversity index( X ± S )</th>
<th>HCC</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chao1</td>
<td>175.58 ± 42.66</td>
<td>216.39 ± 46.49</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ACE</td>
<td>175.71 ± 40.31</td>
<td>217.71 ± 44.36</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Shannon</td>
<td>3.96 ± 0.09</td>
<td>4.71 ± 0.84</td>
<td>0.001</td>
</tr>
<tr>
<td>Simpson</td>
<td>0.86 ± 0.13</td>
<td>0.91 ± 0.07</td>
<td>0.036</td>
</tr>
</tbody>
</table>

The principal coordinates analysis (PCoA), mainly includes Bray-Curtis and UniFrac, was used for Beta diversity analysis. The results of Bray-Curtis indicated that the explanatory variations of two main coordinates PC1 and PC2 were 37.79% and 11.39% respectively (Fig. 7A). The unweighted UniFrac 31.37% and 18.25% respectively (Fig. 7B).

**Prediction of floras metabolic function based on high-quality Sequences**

PICRTUS package was used to predict the floras metabolic function at phylum level. The abundance of genes encoding membrane transport, carbohydrate metabolism and amino acid metabolism were higher than other genes in both groups. The abundance of genes in encoding translation, signal transducing, transcription, replication and repair, transmembrane transport, nucleotide metabolism, amino acid metabolism, lipid metabolism, energy metabolism and carbohydrate metabolism in the healthy control group was higher than that in the HCC group, suggesting that the differences in intestinal flora between may lead to metabolic changes (Fig. 8).

**Liver function index and the levels of interleukin**

According to the above results and relevant studies, we analyzed the possible effects of 19 species whose abundance was different between two groups and concluded that the differences of OTUs between HCC group and control group may cause changes in liver function index and interleukin levels. Serum samples were collected for testing liver function index and interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-12 (IL-12). The results indicated that differences of TP, ALB, TBIL, DBIL, IBIL, TBA, ALT and AST were significant in two groups (Table 2). And the IL-6 (p < 0.001) and IL-10 (p < 0.001) were significant different in two groups, while the difference of IL-12 (p = 0.123) had no statistical significance (Table 3, Fig. 9).
Table 2
Differences in clinical variables of liver function between the two groups (X ± S)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HCC</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP</td>
<td>73.03 ± 4.14</td>
<td>67.60 ± 7.94</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ALB</td>
<td>47.96 ± 2.85</td>
<td>38.94 ± 4.56</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>GLO</td>
<td>25.08 ± 3.62</td>
<td>28.66 ± 7.56</td>
<td>0.029</td>
</tr>
<tr>
<td>TBA</td>
<td>3.69 ± 2.54</td>
<td>28.87 ± 44.24</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>TBIL</td>
<td>11.99 ± 3.74</td>
<td>22.23 ± 10.78</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>DBIL</td>
<td>4.80 ± 1.56</td>
<td>8.70 ± 6.21</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IBIL</td>
<td>7.17 ± 2.30</td>
<td>13.53 ± 5.91</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ALT</td>
<td>16.05 ± 7.76</td>
<td>42.22 ± 26.45</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>AST</td>
<td>18.87 ± 4.88</td>
<td>49.77 ± 23.86</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 3
Expression of three kinds of IL in serum of two groups (X ± S)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HCC</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>2.06 ± 0.60</td>
<td>6.71 ± 1.85</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL-10</td>
<td>7.64 ± 1.67</td>
<td>11.53 ± 4.16</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL-12</td>
<td>7.06 ± 3.38</td>
<td>6.24 ± 3.42</td>
<td>0.321</td>
</tr>
</tbody>
</table>

Correlation analysis between floras and interleukin levels

Among all floras that differ between the two groups, there were 15 colonies and 12 species showed statistical significance to the liver function index or the levels of interleukin. At genus level, Coriobacterium, Atopobium and Coprococcus were related to IL-6 (all |r| ≥ 0.30, all p < 0.05) and IL-10 (all |r| ≥ 0.30, all p < 0.05), results were shown in Fig. 10A. And at species level, V.dispar was related to the IL-6 (r = 0.38, p < 0.001) and IL-10 (r = 0.46, p < 0.001). However, there was no flora showed significant association with IL-12 (Fig. 10B).

Discussion

The study was concentrated on the differences of intestinal floras between HCC patients and health people, and to explain how the differences affected the clinical processes of the HCC, we analyzed the possible differences in metabolites caused by different floras. At Phylum level, compared to the control group, the Cyanobacteria, Proteobacteria and TM7 showed higher abundance in HCC group, while
Firmicutes and Tenericutes showed lower abundance. According to other studies, Proteobacteria and TM7 were reported to be related to other diseases, such as the Proteobacteria could cause inflammation of the gut, and TM7 was higher in Schistosomiasis japonica patients, Firmicutes had a well abundance among the obese people. However, it was interesting that Cyanobacteria, which had a good effect on anti-tumor, should normally have a low abundance in the HCC group, and we concluded that this may be due to the compensatory elevation of Cyanobacteria caused by the occurrence of tumor. It should be noted that the above situation may be caused by insufficient sample size. At genus level, it was found that the abundance of the Actinomycetes, Faecalibacterium and Bacteroides differed between the HCC group and control group.

Based on the above results, we found that there were indeed differences in liver function level, IL-6 and IL-10 expression level between the two groups, but IL-12 showed no statistical significance. The analysis suggested that the changes of intestinal floras may be related to the changes of interleukin levels. IL-6 is a typical cytokine to maintain homeostatic. When the body is damaged by infection or tissue damage, IL-6 will be produced immediately, and IL-6 can stimulate and activate the proliferation of B cells, secrete antibodies, and participate in inflammatory response. Study found that co-culture of hepatic stellate cells and hepatic stellate cells (HSC) could provide the invasion and migration ability of tumor cells, and after the intervention of IL-6, recombinant signal transducer and activator of transcription 3 (STAT3) was activated, and the viability of tumor cells was significantly reduced, and the ability of invasion and migration was weakened. Related studies had shown that significant IL-6 immune reactivity was found in tonsil tissues around actinomycetes colonies. IL-10, as a key immunomodulatory cytokine, may affect T cell and dendritic cell (DC) production, and regulate dendritic cell function by reducing the secretion of surface markers and inflammatory cytokines. Study had shown that IL-10 alleviates liver fibrosis in mice through TGF-β/Smad signaling pathway and is a protective factor for liver fibrosis, which can indirectly serve as a protective factor for HCC. And intestinal floras might influence IL-10 synthesis by influencing propionate production. IL-12 had also been shown to be associated with liver cancer and has strong pro-inflammatory effects, including promotion of effective T cell activation and proliferation-mediated cellular immunity by natural killer cell (NK). Zhou found that IL-12 intervention significantly reduced tumor volume in mouse HCC model. Related literature had shown that some lactic acid bacteria strains can secrete IL-12 by stimulating macrophages and dendritic cells. But the detailed mechanism still needs to be further verified by biological experiments. In addition, some studies suggested that the abundance of Lactobacilli may be related to the expression level of IL-12. However, in our study, only at the order level, there was a statistical difference in the Lactobacilli between the HCC group and the control group, which may be caused by the relatively insufficient sample size.

The study have shown that phylum Verrucomicrobia was decreased in early HCC. At the genus level, 12 genera, including Alistipes, Phascolarctobacterium and Ruminococcus were significantly decreased, while 6 genera, including Klebsiella and Haemophilus were increased in early HCC versus healthy people. The study have shown that IL-25 induced by intestinal dysbiosis promotes the development of HCC.
through selective activation of macrophages in the tumor microenvironment\textsuperscript{[61]}. In addition, imbalance of intestinal bacterial products and flora can promote the progression of cirrhosis and HCC.

With the deepening of research on the relationship between intestinal flora and liver disease, fecal transplantation and probiotics provided new ideas for the treatment of liver disease and even liver cancer. Li found that Prohep, a new probiotic mixture, could effectively reduce the growth of liver tumors in mice by 40\%, especially when probiotics were used before tumor cells were injected, and the probiotic mixture could produce stronger anti-tumor effects by reducing IL-17 and other angiogenic factors\textsuperscript{[62]}. This also confirmed that intestinal flora promotes liver cancer through interleukin. Studies have found that fecal transplantation has a significant effect on patients with chronic liver disease\textsuperscript{[63]}. A single blind trial in a Chinese population showed that after long-term antiviral therapy, fecal transplantation could still induce HBeAg clearance in a considerable number of persistent HBeAg-positive cases, suggesting that altering the intestinal microecology may provide new ideas for the treatment of chronic hepatitis B, and even its possible progression to HCC\textsuperscript{[64]}.

**Conclusion**

The abundance of intestinal floras in the HCC group was different from the control group. Additionally, in HCC patients, the expression levels of IL-6 and IL-10 were higher than health people, and this difference showed a strong correlation to the abundance of some intestinal floras.

**Abbreviations**
<table>
<thead>
<tr>
<th>Full name</th>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>albumin</td>
<td>ALB</td>
</tr>
<tr>
<td>alanine transaminase</td>
<td>ALT</td>
</tr>
<tr>
<td>aspartate transferase</td>
<td>AST</td>
</tr>
<tr>
<td>body mass index</td>
<td>BMI</td>
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<tr>
<td>direct bilirubin</td>
<td>DBIL</td>
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<tr>
<td>dendritic cell</td>
<td>DC</td>
</tr>
<tr>
<td>globulin</td>
<td>GLO</td>
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<tr>
<td>hepatocellular carcinoma</td>
<td>HCC</td>
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<tr>
<td>hepatic stellate cells</td>
<td>HSC</td>
</tr>
<tr>
<td>indirect bilirubin</td>
<td>IBIL</td>
</tr>
<tr>
<td>interleukin</td>
<td>IL</td>
</tr>
<tr>
<td>microbial associated molecular pattern</td>
<td>MAMP</td>
</tr>
<tr>
<td>natural killer cell</td>
<td>NKC</td>
</tr>
<tr>
<td>operational taxonomic units</td>
<td>OTUs</td>
</tr>
<tr>
<td>the principal coordinates analysis</td>
<td>PCoA</td>
</tr>
<tr>
<td>signal transducer and activator of transcription 3</td>
<td>STAT3</td>
</tr>
<tr>
<td>total bile acids</td>
<td>TBA</td>
</tr>
<tr>
<td>total bilirubin</td>
<td>TBIL</td>
</tr>
<tr>
<td>total protein</td>
<td>TP</td>
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</tbody>
</table>

**Declarations**

**Ethics approval and consent to participate**

Our manuscript was approved by the ethics Committee of Longhua District People's Hospital of Shenzhen. The committee’s reference number is 2021.054. We have provided a supplementary document for Ethics Approval and Informed Consent. And we confirmed that the informed consent has been taken for "Consent to participate".

**Consent for publication**

We obtained the consent of the patients and have a copy in Chinese. Written instructions can be provided during the follow-up process if required.
Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

Funding

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Authors' contributions

WQX provided the experimental ideas, collected samples and was a major contributor in writing the manuscript.

YJ Contacted the person in charge of the institution and the patient.

JLT analyzed the experimental data.

HYL tested stool sample.

WYX tested stool sample.

CC reviewed literature and summarized data.

XXJ applied for the project funding and guided the author WQX to write articles.

All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References


13(9): e0203657.

Figures
Figure 1. Map of 16s rRNA sequencing. (A) 4,976,854 available readings from 88 samples. (B) Venn map of OTUs, HCC group in yellow, control in blue, and overlapping part in cyan. (C) constituent ratio for abundance of OUTs for each samples from kingdom to species and unclassified included.
Figure 2. Curves used to indicate sufficient depth of sequencing. (A) Rarefaction curve: Each curve represents one sample, and the X-axis represents sequence reading number, and the Y-axis represents OTU number. (B) Species accumulation curve: Measure and predict how much species abundance increases with sample size.

Figure 2

See image above for figure legend
Figure 3. The violin figure showed different abundance between HCC group and control group at the phylum level. The abscissa is the grouping information, the ordinate is the sequence quantity of each taxon in each group, and a single rectangular box is a single taxon. The violin diagram can intuitively display the distribution characteristics of data. The fatness and thinness of "violin" reflects the density of sample data distribution (the wider the width, the more samples corresponding to the sequence quantity). The boxplot border represents the Interquartile range (IQR), the horizontal line represents the median value, the upper and lower whiskers respectively represent the 1.5 times IQR range beyond the upper and lower quartile, and the symbol "•" represents the extreme value beyond the range.

Figure 3

See image above for figure legend
Figure 4. The violin figure showed different abundance between HCC group and control group at the special level (the same type as Figure 3). There were statistically significant differences in the abundance of 19 species between the two groups, with all \( P < 0.05 \).
Figure 5. LEfSe analysis was used to screen for key community members by Kruskal-Wallis test and Wilcoxon test. (A) Classification tree shows the sample population from the phylum to the genus (from the inner circle to the outer ring lined up) all taxa of hierarchy, node size corresponds to the average relative abundance of the taxa, yellow nodes represent did not show significant difference between group of taxa, and other color (e.g., green and red) shows that these taxa showed significant differences between groups, And the color represented in the grouping sample abundance is higher. Letters identify taxon names with significant differences between groups. (B) The ordinate represents the taxa with significant differences between groups, and the horizontal coordinate shows the logarithmic score of LDA difference analysis for the corresponding taxa intuitively by a bar graph. They are sorted according to the size of score values to describe their differences in different groups of samples. The longer the length, the more significant the difference of the taxon, and the different colors of the bar chart indicate the group of samples with higher abundance corresponding to the taxon.

Figure 5

See image above for figure legend
Figure 6. Boxplots of four indexes. The boxplot border represents the Interquartile range (IQR), the horizontal line represents the median value, the upper and lower whiskers respectively represent the 1.5 times IQR range beyond the upper and lower quartile, and the symbol "•" represents each data beyond the range. (A) Chao1 index. (B) Ace index. (C) Simpson index. (D) Shannon index.

Figure 6

See image above for figure legend
Figure 7. PCoA and unweighted UniFrac were used to explain variations between groups. (A) 2D plot of PCoA: The abscissa PC1 represents the richest dimension (variation explained 37.79%), and the ordinate PC2 indicates the second richest dimension (variation explained 11.39%), each point represents the position of the sample in the PC1-PC2 coordinate system. (B) Box plot of PCoA: The abscissa represents grouping factors, and the ordinate represents the relative distances of samples from different groups. (C) 2D plot of unweighted UniFrac: The abscissa PC1 represents the richest dimension (variation explained 31.37%), and the ordinate PC2 indicates the second richest dimension (variation explained 18.25%), each point represents the position of the sample in the PC1-PC2 coordinate system. (D) Box plot of unweighted UniFrac: The abscissa represents grouping factors, and the ordinate represents the relative distances of samples from different groups.
Figure 8. Boxplots of metabolic-related gene categories and abundance, at phylum level, according to the KEGG database. The boxplot border represents the Interquartile range (IQR), the horizontal line represents the median value, the length of the boxplot indicated the abundance of the gene. The abundance of genes in encoding translation, signal transducing, transcription, replication and repair, transmembrane transport, nucleotide metabolism, amino acid metabolism, lipid metabolism, energy metabolism and carbohydrate metabolism show different in two groups.

Figure 8

See image above for figure legend
Figure 9. Comparison of interleukin levels between two groups. (A) Concentration of IL-6 between two groups, p<0.001. (B) Concentration of IL-10 between two groups, p<0.001. (C) Concentration of IL-12 between two groups, p=0.321.

Figure 9

See image above for figure legend
Figure 10. Heat map to show the relationship between intestinal flora and clinical variables at the genus level and the species. (A) Heat map for Spearman correlation analysis between intestinal flora and clinical variables at the genus level, X-axis: clinical variables, Y-axis: genus. The branches of the figure on the left indicates the classification of phylum, R-values are shown in different colors in the heat map. (B) Heat map for Spearman correlation analysis between intestinal flora and clinical variables at the species level.

Figure 10

See image above for figure legend