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ORIGINAL ARTICLE

Analysis of the intestinal flora in patients with primary liver cancer*

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Abstract

Objective To investigate the differences in intestinal flora of patients with primary liver cancer and of healthy individuals and to investigate the effect of the differential flora on the development of liver cancer.

Methods Overall, 67 patients with primary liver cancer who received systematic and complete treatment between January 2019 and December 2020 at the Sixth People's Hospital of Qingdao and had complete clinical data were enrolled in this study, and 26 individuals who were healthy on physical examination in the same period were used as healthy controls. Macro genome and 16s ribosome Deoxyribo Nucleic Acid (rDNA) high-throughput sequencing were performed on the stool flora of the enrolled patients and controls, and the differences in the intestinal flora were analyzed using the LEfSe bioinformatics software.

Results Compared with the control samples, all the tested patient samples showed statistically significant differences in the number of colonies of 5 bacterial phyla, 5 orders, 8 families, 11 genera, and 14 species ($P < 0.05$).

Conclusion Compared with healthy people, patients with primary liver cancer have significant differences in the intestinal flora composition. The alteration of the intestinal flora may be correlated with the occurrence of primary liver cancer, and the intestinal flora may become a novel target for the prevention and treatment of primary liver cancer.

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Key words: liver cancer; intestinal flora; genome sequencing; 16s rDNA

Introduction flora is abundant and diverse, and it has an indispensable role in human digestion, metabolism, defense, and immunity. The intestinal flora is involved in the development and progression of many diseases [1]. Inflammatory diseases (e.g., enteritis and pancreatitis), metabolic diseases (e.g., type 2 diabetes mellitus and obesity), and psychiatric diseases (e.g., depression and Alzheimer's disease) are closely related to changes in the intestinal flora [2–4]. Primary hepatocellular carcinoma (HCC) is one of the most common malignancies and poses a serious risk to human health. Evidence suggests that intestinal flora composition is strongly associated with primary HCC, and that the intestinal microbiota plays a key role in promoting the progression of liver disease and HCC development. At present, in the diagnosis and treatment of liver-related diseases, most clinicians do not know enough about the correlation between the intestinal flora and liver diseases and do not consider treating liver diseases by regulating intestinal flora dysbiosis; therefore, a systematic and detailed exploration of intestinal flora changes in liver cancer patients can help us find new ways to prevent and treat liver cancer, achieve early diagnosis, and target treatment to improve prognosis.

Materials and methods

General information

Overall, 26 individuals who were healthy on physical examination at the Sixth People's Hospital of Qingdao between January 2019 and December 2020 were recruited as healthy controls (Group A), and 77 patients with liver cancer who received systematic and complete treatment

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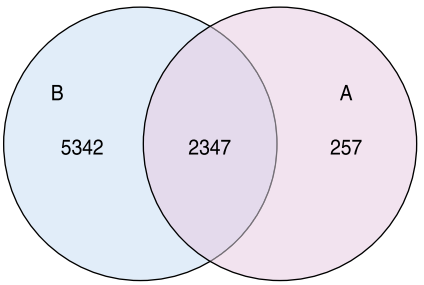

Fig. 1 Number of OTUs for both groups

Table 1 Differences in the phylum-level flora between the two groups

Group	A	B	*t*/*Z*	*P*
Bacteroidetes	50.52 ± 17.71	41.26 ± 14.36	2.75	< 0.01
Frimicutes	37.28 ± 17.49	43.87 ± 14.49	–1.85	0.07
Proteobacteria	5.80 (2.27, 11.96)	5.91 (3.50, 10.14)	–0.547	0.585
Actinobacteria	0.65 (0.41, 2.37)	1.59 (0.60, 3.20)	–1.549	0.121
Tenericutes	0.26 (0.05, 1.07)	0.14 (0.06, 0.39)	–1.253	0.210

during the same period (Group B) with complete clinical data were included in this study. The inclusion criteria for patients with liver cancer were as follows: disease diagnosis in accordance with the diagnostic criteria of the Diagnostic Code for Primary Liver Cancer (2017 version) [5]; normal function of vital organs such as the heart, brain, and kidney; no previous history of liver surgery; no liver transplantation; and no use of antibacterial or other drugs affecting the intestinal flora in the 3 months prior to enrollment. The inclusion criteria for the controls were as follows: no intestinal disease within the past 3 months and no use of antibiotics or other drugs that affect the intestinal flora within the past 3 months. The exclusion criteria for liver cancer patient group were as follows: autoimmune, drug-induced, or parasitic liver disease, human immunodeficiency virus infection, malnutrition, and a history of psychosis or psychiatric disorders. There was no statistical difference between the groups in terms of gender, age, disease duration, and other general information, and they were comparable. The study was ethically reviewed, and all patients included in this study were informed and agreed to participate voluntarily.

Sample collection and index testing

Stool samples (≥10 g) were collected from participants with sterile swabs, placed in sterile containers containing cache solution, and stored at -80°C within 1 h. The stool flora of both the patients and controls were subjected to 16s rDNA high-throughput and macro genome sequencing. Thereafter, the flora diversity and the flora differences were analyzed using LEfSe bioinformatics software.

Table 2 Differences in the class-level flora between the two groups

Group	A	B	*t*/*Z*	*P*
Bacteroidia	50.72 ± 17.36	41.19 ± 14.37	2.77	< 0.01
Bacilli	0.70 (0.32, 0.90)	1.87 (0.86, 3.22)	–4.21	< 0.01
Betaproteobacteria	2.12 (1.26, 4.34)	1.48 (1.05, 2.02)	–2.29	0.02
Erysipelotrichi	0.35 (0.18, 0.48)	0.82 (0.46, 1.51)	–4.73	< 0.01
Coriobacteriia	0.15 (0.10, 0.33)	0.37 (0.18, 0.84)	–2.63	< 0.01

Statistical methods

The data were processed and analyzed using SPSS software SPSS25.0, and the data are expressed as means ± standard deviations and medians (quartiles). Data were analyzed using the independent samples *t*-test and rank sum test. Statistical significance was set at $P < 0.05$.

Results

Differential analysis of Operational Taxonomic Unit (OTU) expression numbers

Overall, 7,689 OTUs were detected in the patient with liver cancer group and 2,604 OTUs were in the control group. Of these OTUs, 2,347 were common to both groups, 5,342 were unique to the patients with liver cancer, and 257 OTUs were unique to the controls (Fig.1).

Comparison of the relative abundance of the intestinal flora at the phylum level

Comparison of the relative abundance of liver cancer patients and healthy individuals at the phylum level showed that the top five significantly different phyla were Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Tenericutes. Only one microbial phylum, Bacteroidetes, showed significant statistical differences, with its abundance in the gut microbiota of the liver cancer group significantly lower than that of the healthy control group ($P < 0.05$; Table 1).

Comparison of the relative abundance of the intestinal flora at the class level

When comparing the relative abundance of the intestinal flora at the class level between the patients with liver cancer and controls, the following were the top five classes with a significantly different abundance: Bacteroidia and Betaproteobacteria had a decreased abundance, whereas Bacilli, Coriobacteriia, and Erysipelotrichia had an increased abundance. All differences were statistically significant ($P < 0.05$; Table 2).

Table 3 Differences in the order-level flora between the two groups

Group	A	B	*t*/*Z*	*P*
Bacteroidales	50.72 ± 17.36	41.19 ± 14.37	2.77	< 0.01
Burkholderiales	2.09 (1.23, 4.33)	1.37 (0.84, 1.98)	–2.60	< 0.01
Lactobacillales	0.46 (0.20, 0.74)	1.45 (0.63, 2.50)	–3.89	< 0.01
Erysipelotrichales	0.35 (0.17, 0.48)	0.82 (0.45, 1.51)	–4.73	< 0.01
coriobacteriales	0.14 (0.10, 0.33)	0.38 (0.18, 0.84)	–2.62	< 0.01

Comparison of the relative abundance of the intestinal flora at the order level

When comparing the relative abundance of the intestinal flora at the order level in the patients with liver cancer and controls, the following were the top five orders with a significantly different abundance: Bacteroidales had a decreased abundance, whereas Burkholderiales, Lactobacillales, Erysipelotrichales, and Coriobacteriales had an increased abundance. All differences were statistically significant ($P < 0.05$; Table 3).

Comparison of the relative abundance of the intestinal flora at the family level

When comparing the relative abundance of the intestinal flora at the family level in the patients with liver cancer and controls, the following were the top eight families that showed a significantly different abundance: *Alcaligenaceae*, *S247*, *Barnesiellaceae*, and *Christensenellaceae* had a decreased abundance, whereas *Coriobacteriaceae*, *Streptococcaceae*, *Erysipelotrichaceae*, and *Enterococcaceae* had an increased abundance. All differences were statistically significant ($P < 0.05$; Table 4).

Comparison of the relative abundance of the intestinal flora at the genus level

When comparing the relative abundance of the intestinal flora at the genus level between patients with liver cancer and controls population, the following were the top 11 genera with a significantly different abundance: *Roseburia*, *Ruminococcus*, *Clostridium*, *Streptococcus*, *Collinsella*, *Megamonas*, Veillonella, *Eubacterium*, *Enterococcus*, and *Pseudobutyrivibrio* had an increased abundance, *whereas Sutterella* had a decreased abundance. All differences were statistically significant ($P < 0.05$; Table 5).

Comparison of the relative abundance of the intestinal flora at the species level

When comparing the relative abundance at the species level between patients with liver cancer and controls, the following were the top 14 species with a significantly difference abundance: *Ruminococcus gnavus*, *Dorea formicigenerans*, *Collinsella aerofaciens*, *Veillonella dispar*, *Bacteroides plebeius*, *Streptococcus infantis*, *Ruminococcus torques*, *Eubacterium dolichum*, *Veillonella parvula*, and *Clostridium ramosum* had an increased abundance, whereas *Bacteroides coprophilus*, *Bacteroides eggerthii*, *Bifidobacterium bifidum*, and *Lactococcus garvieae* had a decreased abundance. All differences were statistically significant ($P < 0.05$; Table 6). Genus dendrogram of mycorrhizal species (Fig. 2).

Discussion

Primary liver cancer is usually associated with chronic liver disease (e.g., hepatitis, cirrhosis, and steatohepatitis), and its development is typically accompanied by an inflammatory response. Microecological studies are receiving increasing attention from both researchers and clinicians. Currently, patients with an early diagnosis of HCC can be treated with transcatheter arterial chemoembolization and radiotherapy; however, many patients are diagnosed at an intermediate to advanced stage and often present with a poor prognosis. Therefore, early prevention and diagnosis are the key to treating liver cancer. The intestinal flora is involved in the maintenance of homeostasis in the body and is associated with a variety of pathophysiological processes; the liver is one of the organs that is most susceptible to the effects of the intestinal flora [6]. Previous studies have confirmed a strong association between the intestinal flora and liver cancer. Therefore, alteration of the intestinal flora may be one of the underlying mechanisms of liver cancer development. Exploring the unique characteristics of the intestinal flora in patients with primary liver cancer is expected to enable early prevention and diagnosis of liver

Table 4 Differences in the family-level flora between the two groups

Group	Alcaligenaceae	S247	Barnesiellaceae	coriobacteriaceae	streptococcaceae	Erysipelotrichaceae	Enterococcaceae	Christensenellaceae
A	2.09 (1.20, 3.80)	0.47 (0.24, 1.48)	035 (0.15, 0.59)	0.15 (0.10, 0.33)	0.18 (0.09, 0.30)	0.35 (0.17, 0.47)	0.02 (0.01, 0.04)	0.17 (0.03, 0.39)
B	1.21 (0.68, 1.70)	0.14 (0.08, 0.29)	0.14 (0.06, 0.29)	0.38 (0.18, 0.84)	0.66 (0.21, 1.65)	0.82 (0.46, 1.51)	0.04 (0.02, 0.14)	0.04 (0.02, 0.11)
Z	–3.12	–3.81	–2.90	–2.62	–3.94	–4.73	–3.32	–2.87
P	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

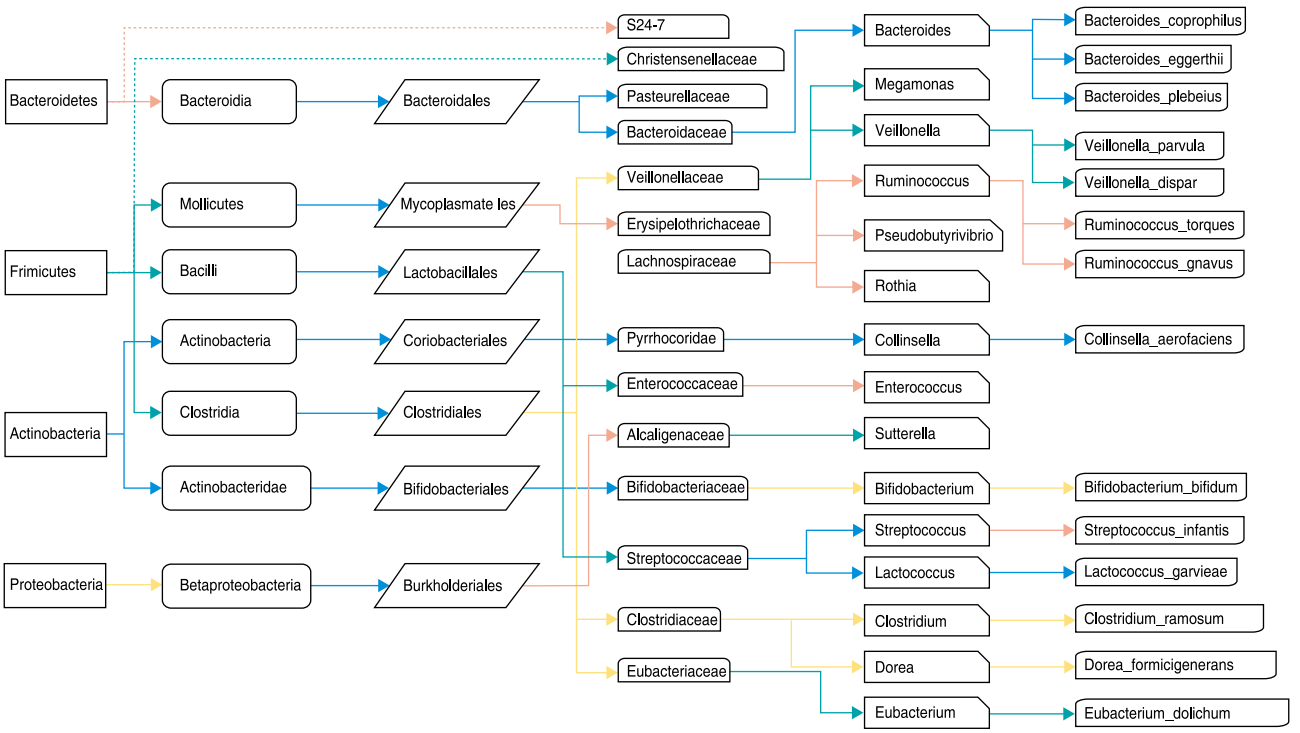

Fig. 2 Species relationship

Table 5 Differences in the genus-level flora between the two groups

Group	A	B	Z	P
Sutterella	2.09 (1.20, 3.79)	1.21 (0.68, 1.69)	–3.16	< 0.01
Roseburia	3.01 (1.89, 5.39)	5.24 (2.59, 7.43)	–2.44	0.01
Ruminococcus	1.81 (1.17, 3.45)	3.41 (2.12, 4.85)	–3.11	0.01
Clostridium	0.64 (0.41, 1.09)	0.87 (0.46, 2.05)	–2.07	0.04
Streptococcus	0.16 (0.09, 0.25)	0.63 (0.20, 1.47)	–4.30	< 0.01
Collinsella	0.08 (0.04, 0.22)	0.21 (0.07, 0.56)	–2.37	0.02
Megamonas	0.05 (0.01, 0.19)	0.16 (0.06, 0.42)	–2.38	0.02
Veillonella	0.04 (0.02, 0.05)	0.25 (0.06, 0.61)	–4.904	< 0.01
Eubacterium	0.06 (0.02, 0.11)	0.14 (0.07, 0.28)	–2.991	< 0.01
Enterococcus	0.01 (0.01, 0.03)	0.04 (0.02, 0.14)	–3.826	< 0.01
Pseudobutyrivibrio	0.00 (0.00, 0.01)	0.02 (0.01, 0.05)	–4.334	< 0.01

cancer, and early intervention regarding an abnormal flora may delay the disease process and provide new insights for the prevention and treatment of primary liver cancer.

Gut-liver axis and HCC correlation

The concept of the "gut-liver axis" has received much attention in recent years [7], and the relationship between the intestinal flora and liver disease has been redefined. The liver receives blood from the portal vein and nutrients absorbed by the intestine, as well as pathogenic bacteria and metabolites. An imbalance in the intestinal flora impairs the intestinal barrier and immune status, and pathogenic bacteria and flora metabolites are more likely to enter the liver through the portal vein and participate in pathophysiological processes in the liver, causing or promoting the development of liver disease [8]. *Veillonella parvula* is an anaerobic opportunistic pathogenic bacterium that is parasitic in the oral cavity and intestine and has been reported to cause bacteremia, meningitis, endocarditis, prosthetic joint infections, pulmonary infections, and vertebral osteomyelitis after spreading to other parts of the body [9, 10]. A previously published article demonstrated that *Bacteroides plebeius* plays a role in the degradation of porphyrins [11]. In this study, the abundance of common Bacteroides in the gut of liver cancer patients was significantly increased, which may indicate excessive degradation of porphyrins. *Bifidobacterium bifidum* produces biologically active interleukin 10 [12] and can inhibit the development of inflammation by targeting toll-like receptors via NF-κB [13]. This has also been shown to improve symptoms in patients with Alzheimer's disease [14] and to inhibit tumor growth by acting in concert with programmed cell death protein 1 (PD-1) inhibitors to induce host antitumor immune responses [15]. On sequencing the flora of patients with HCC, a decreased abundance of *Bifidobacterium bifidum* was observed, leading to decreased nutrient absorption and impaired immune function. *Ruminococcus gnavus* has been found to be enriched in patients with inflammatory bowel disease; its capsular polysaccharide promotes local inflammatory immune responses while activating hepatic oxidative

Table 6 Differences in the species-level flora between the two groups

Group	A	B	*Z*	*P*
Bacteroides_coprophilus	0.08 (0.04, 0.16)	0.01 (0.00, 0.14)	–2.67	< 0.01
Ruminococcus_gnavus	0.31 (0.22, 0.65)	0.68 (0.31, 1.27)	–2.97	< 0.01
Bacteroides_eggerthii	0.15 (0.06, 0.50)	0.06 (0.02, 0.25)	–2.69	< 0.01
Dorea_formicigenerans	0.04 (0.02, 0.12)	0.09 (0.05, 0.13)	–2.26	0.02
Collinsella_aerofaciens	0.10 (0.04, 0.20)	0.20 (0.06, 0.55)	–2.01	0.04
veillonella_dispar	0.02 (0.01, 0.03)	0.18 (0.05, 0.51)	–5.09	< 0.01
Bifidobacterium_bifidum	0.01 (0.00, 0.07)	0.00 (0.00, 0.01)	–3.95	< 0.01
Bacteroides_plebeius	0.43 (0.08, 2.51)	2.08 (0.59, 9.35)	–2.99	< 0.01
streptococcus _infantis	0.02 (0.01, 0.03)	0.06 (0.03, 0.11)	–4.66	< 0.01
Ruminococcus_torques	0.02 (0.01, 0.06)	0.05 (0.02, 0.10)	–2.49	0.01
Eubacterium_dolichum	0.01 (0.00, 0.03)	0.05 (0.01, 0.10)	–3.60	< 0.01
veillonella_parvula	0.01 (0.00, 0.02)	0.06 (0.02, 0.15)	–4.39	< 0.01
Lactococcus_garvieae	0.02 (0.01, 0.06)	0.00 (0.00, 0.01)	–3.96	< 0.01
Clostridium_ramosum	0.01 (0.00, 0.01)	0.03 (0.01, 0.05)	–4.10	< 0.01

stress [16, 17] and can even lead to bacteremia in patients with hematological malignancies [18]. *Veillonella dispar* is commonly found in the oral cavity, but there are reports indicating that bladder cancer patients may develop bacteremia due to intestinal Veillonella_dispa infection [19, 20]. All of the differentially abundant bacterial taxa mentioned above have a direct or indirect relationship with liver function.

Effect of metabolic diseases on liver cancer

In recent years, with changes in living standards and diet structure, disorders of glucose metabolism and fatty liver disease, apart from hepatitis and cirrhosis, have become high-risk factors for the development of HCC. In addition, disorders of hepatic lipid metabolism and the interruption of dynamic balance lead to the accumulation of lipids in hepatocytes and to hepatocellular steatosis. The incidence of fatty liver disease in China is gradually increasing and has become the second most common liver disease after viral hepatitis. Many scholars believe that fatty liver disease is closely related to HCC [21]. *Clostridium ramosum* has been shown to cause obesity and affect liver metabolism in mouse models [22]; *Ruminococcus torques* is significantly more abundant in obese populations [23]; and *Dorea formicigenerans* is positively correlated with obesity and can be used as an indicator of obesity [24]. Moreover, the abundance of *Eubacterium dolichum* has been demonstrated to be increased when rats are fed a high-sugar and high-fat diet decreased with the addition of flaxseed to the diet, indicating that *Eubacterium dolichum* is enriched in the intestine when an unhealthy dietary structure is present [25]. The sequencing results showed that the abundance of all four of these obesity-related bacteria was significantly increased in the intestinal flora of patients with liver cancer. In recent years numerous studies have shown that *Bifidobacterium bifidum* has an important contribution to physical health and can promote the digestion of food and the absorption of nutrients [26]. In addition, the results of animal experiments have shown that the addition of *Bifidobacterium bifidum* can effectively relieve constipation [27]. Despite these health advantages, the abundance of *Bifidobacterium bifidum* is significantly reduced in patients with HCC, which is detrimental to the health of these patients.

The relationship between the intestinal flora and cancer

Some studies have reported that the structure of the intestinal flora is closely associated with pancreatic, colon, thyroid, and liver cancers, and by reviewing the literature, 3 of the top 14 flora with significant differences in the results of this experiment were correlated with malignancy. Notably, *Bacteroides eggerthii* can produce antitumor compounds using quercetin [28]. The results suggest that its abundance was reduced in patients with HCC, which is similar to the findings of other studies on patients with colon cancer, where the bacterium was also significantly reduced [29]. *Streptococcus infantis* was shown to be closely associated with oral cancer when its abundance increased [30]. In recent years, anti-PD-1 therapy for tumors has become a hot research topic, but not all patients can benefit from it. The higher abundance of *Collinsellaaerofaciens* in the intestinal flora of patients in whom anti-PD-1 therapy was effective was verified in animal experiments, and transplantation of PD-1-sensitive patient flora enhanced T cell responses and improved the efficacy of anti-PD-L1 therapy [31]. Furthermore, the

increased abundance of this bacterium in the intestinal flora of patients with liver cancer suggests that they could benefit more from PD-L1 blockade therapy.

Intestinal flora is regulated by genetic factors

The structure of the intestinal flora is influenced by many factors, including dietary habits, diseases, and the application of antibiotics. Genetics also plays an important role in shaping the structure of the flora. As early as 2001, Zoetendal et al. demonstrated a high degree of similarity in the gut flora of twins using DNA techniques [32]. In 2014, Goodrich *et al.* [33] found that the abundance of many bacteria in the intestinal flora was influenced by the genetic background of the host, and that the gut flora of identical twins was more similar than that of heterozygous twins. Christensenellaceae was also shown to be the most heritable bacterium and to form symbiotic networks with other heritable bacteria, thereby influencing host metabolism. Yatsunenko later verified Goodrich's experimental results using more samples and found that bacteria of the phylum Synechococcus were non-heritable and were mainly influenced by environmental factors, whereas the thick-walled Actinomycetes and soft-walled Archaea phyla were heritable [34, 35]. The abundance of *Christensenellaceae* in patients with liver cancer in this experimental study was significantly lower than that in controls. This indicates that there may be a correlation between this flora and liver cancer susceptibility genes, which can be assessed by screening the intestinal flora of people at high risk of developing liver cancer.

In summary, compared with healthy individuals, patients with primary liver cancer have significantly altered intestinal flora, and patients with liver cancer can be evaluated by monitoring changes in the intestinal flora.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Chengcong Liu wrote this case report and analyzed all the data. Guoxin Sun collected the literature for this paper. Chengcong Liu, Guoxin Sun, Huizhe Wang, and Gaishuang Shang completed the operation. Xiong Yan and Xiao Zou validated modifications to the paper. Xiao Zou provided financial support for the article's writing and publication. All the authors have read and approved the final manuscript.

Data availability statement

Not applicable.

Ethical approval

The study was ethically reviewed, and all study participants provided informed consent and participated voluntarily.

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