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Association of disease condition with changes in intestinal flora, and plasma endotoxin and vascular endothelial growth factor levels in patients with liver cancer

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Abstract. – OBJECTIVE: Currently, the therapeutic effect on patients with liver cancer is associated with disease development. Meanwhile, the efficacy in patients with advanced liver cancer is far from satisfactory. Therefore, the aim of this study was to explore the association of disease condition with changes in liver function indexes, intestinal flora, and plasma endotoxin (ET) and vascular endothelial growth factor (VEGF) levels in patients with liver cancer.

PATIENTS AND METHODS: A total of 300 patients with primary liver cancer in our hospital were enrolled in this study. All patients were divided into three groups, including early liver cancer group, middle liver cancer group, and advanced liver cancer group. Peripheral blood was collected from each subject to detect liver function indexes, procalcitonin (PCT), plasma ET, and VEGF levels. Furthermore, mid-posterior-segment stools were collected from 15 cases in each group, and sent to the company for detection of intestinal flora.

RESULTS: Liver function indexes in peripheral blood of patients with liver cancer changed with the changes in disease condition. With the progression of liver cancer, the level of aspartate aminotransferase (AST) increased significantly, and the highest was observed in advanced liver cancer patients [(91.18±10.34) U/L] (p=0.046). However, the level of plasma total protein declined significantly, which was (24.83±1.75) g/L in advanced liver cancer patients (p=0.035). The changes in total bilirubin were significantly associated with the progression of liver cancer (p=0.003). The abundance of Clostridiales, Firmicutes, and Streptococcus in the intestinal tract was high in early liver cancer group. The abundance of Ruminococcaceae, Pasteurellaceae, Tanticharoenia, and Vagococcus in the intestinal tract was high in middle liver cancer group. Meanwhile, the abundance of Bifidobacteriales, Actinobacteria, Barnesiella, Porphyromonadaceae, and Pseudomonadales in the intestinal tract was high in advanced liver cancer group. In patients with liver cancer, the level of Enterobacteriaceae was positively correlated with that of Firmicutes (r=0.36, p=0.003), whereas it was negatively correlated with Lactobacillus (r=-0.72, p=0.021). The level of Lactobacillus was positively correlated with that of Ruminococcaceae (r=0.39, p=0.043), whereas it was negatively correlated with that of Firmicutes (r=-0.27, p=0.019). In addition, the level of PCT markedly rose in advanced liver cancer group [(6.89±0.35) ng/mL] (p=0.021). The level of ET increased significantly with the development of liver cancer, with the highest level observed in advanced liver cancer group [(0.71±0.09) EU/mL] (p=0.004). The level of VEGF also increased remarkably with the aggravation of liver cancer, and the highest was found in advanced liver cancer group [(112.33±2.11) μmol/L], showing differences among groups (p<0.05).

CONCLUSIONS: With the progression of liver cancer, the abundance of Barnesiella, etc., rose and that of Ruminococcaceae, etc., declined in the intestinal tract. Meanwhile, the composition of intestinal flora was changed, and the levels of plasma ET and VEGF increased.

Key Words:
Liver cancer, Intestinal flora, Plasma endotoxin.

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Introduction

Liver cancer seriously threatens people's health in all countries around the world and its incidence rate remains high in every continent. Currently, liver cancer is difficult to be cured[1]. In China, the number of patients with hepatitis B cirrhosis is increasing with the prevalence of chronic hepatitis B virus. This indirectly increases the incidence of liver cancer[2]. The mechanism of liver cancer has not been fully clarified yet. It has only been detected that the molecular mechanism of liver cancer is related to the activation of Wnt/catenin signaling pathway and mitogen-activated protein kinase/extracellular regulated protein kinases (MAPK/ERK) pathway. Meanwhile, it is correlated with the composition and activation state of tumor immune microenvironment[3]. As a digestive tract tumor, liver cancer shows a great correlation with the type and abundance of intestinal microorganisms. The treatment of liver cancer is dominated by surgical resection supplemented by chemotherapy. Researches have demonstrated that its efficacy is affected by the progression of tumor[4].

Intestinal microorganisms refer to various bacterial florae in the human normal digestive tract. Currently, hundreds of species of intestinal microorganisms have been identified. The abundance is related to microorganism species, mainly including *Enterococcocci* and *Enterobacteriaceae*. Intestinal microorganisms have become a research hotspot currently. They have been proved to be able to affect the body's immune state, stress response, and metabolic level[5]. Intestinal microorganisms have been found involved in many diseases. Notably, changes in intestinal flora are associated with gastrointestinal discomfort in asthmatic children[6]. The composition of intestinal microorganisms is related to the occurrence of type 2 diabetes mellitus[7]. Meanwhile, the alteration of intestinal flora will lead to liver fibrosis[8]. It has been proved that intestinal flora in liver cancer patients is highly correlated with tumor progression[9,10]. Therefore, the exploration of intestinal microorganisms in liver cancer patients may help to determine the progression of liver cancer more accurately and to find a better therapeutic regimen at each stage.

In this paper, liver cancer patients who received treatment in our hospital were divided into three groups according to the disease condition, including early liver cancer group, middle liver cancer group, and advanced liver cancer group. Changes in the composition and abundance of intestinal microorganisms were analyzed in each group. Meanwhile, the changes in liver function indexes, and procalcitonin (PCT), plasma endotoxin (ET), and vascular endothelial growth factor (VEGF) levels were detected. Our findings might help to determine the influencing factors for the progression of liver cancer and their associations with the disease condition.

Patients and Methods

General Data

A total of 300 patients with primary liver cancer in our hospital from 2017 to date were enrolled as research subjects. All patients were divided into three groups according to the clinical stage of liver cancer, including early liver cancer group (stage I, n=100), middle liver cancer group (stage II, n=100), and advanced liver cancer group (stage III, n=100). The clinical data of patients, such as name, ID No., gender, age, length of hospital stay, and pathological diagnosis, were recorded. According to statistics, there were no statistical differences in such general data as gender and age among the three groups. Informed consent was obtained from each subject before the study. This investigation was approved by the Ethics Committee of our hospital.

Clinical stage of liver cancer was as follows: Stage I (early liver cancer): maximum diameter of single tumor ≤3 cm, and no cancer embolus, abdominal lymph node and distant metastases, with liver function Child-Pugh class A; the sum of the maximum diameter of single or two tumors ≤5 cm, hemi-hepatic tumor, and no cancer embolus, abdominal lymph node and distant metastases, with liver function Child-Pugh class A. Stage II (middle liver cancer): the sum of the maximum diameter of single or two tumors ≤10 cm, the sum of the maximum diameter of hemi-hepatic or two tumors ≤5 cm, left-right hepatic tumor, and no cancer embolus, abdominal lymph node and distant metastases, with liver function Child-Pugh class A; the sum of the maximum diameter of single or two tumors >10 cm, the sum of the maximum diameter of hemi-hepatic or two tumors >5 cm, left-right hepatic tumor, and no cancer embolus, abdominal lymph node and distant metastases in many tumors, with liver function Child-Pugh class A; regardless of the condition of tumor, there are tumor emboli in the portal branch, hepatic vein or bile duct, with liver function Child-Pugh class B. Stage III (advanced liver cancer): regardless of the

condition of tumor, there are tumor emboli in the main portal vein or inferior vena cava, abdominal lymph node or distant metastasis, with liver function Child-Pugh class A or B; liver function Child-Pugh class C regardless of the condition of tumor, cancer embolus, and metastasis.

Detection of Intestinal Flora

Fresh mid-posterior-segment stools were collected from 15 cases in each group, and cryopreserved in liquid nitrogen for the detection of intestinal flora. Collected specimens were obtained and sent with dry ice to Shanghai Servicebio Co., Ltd. (Wuhan, China) for the analysis of intestinal flora. After microbial genomic DNA extraction, amplification, base-building, and labeling, high-throughput sequencing was performed using Illumina MiSeq and Ion PGM. The species and relative abundance of microorganisms in specimens were detected, followed by bioinformatics analysis.

Detection of Liver Function Indexes

A total of 5 mL of fasting peripheral blood was drawn from patients in each group. After centrifugation at 3500 rpm for 5 min, the color of upper-layer plasma was observed. Subsequently, part of it was aspirated into a new centrifuge tube. The remaining plasma was detected using a full-automatic biochemical analyzer. Finally, the levels of liver function indexes aspartate aminotransferase (AST), alanine aminotransferase (ALT), total protein (TP), albumin (ALB), gamma-glutamyl transpeptidase (GGT), and serum total bilirubin (STB) were obtained. Changes of intestinal barrier in each group were examined by colonoscopy.

Limulus Test

Plasma level of ET was measured using limulus test in the three groups. Firstly, limulus lysate was used to label positive control and negative control samples, and the standards were diluted. Later, ET solution was added into positive control samples, and deionized water was added into negative control samples. Meanwhile, patient's plasma was added into other tubes. Next, the tubes were closed, shaken evenly, and placed vertically in water bath tank at 37°C. After 1 h, the tubes were taken out and observed.

Detection of PCT and VEGF

PCT and VEGF were determined *via* enzyme-linked immunosorbent assay (ELISA) using the R&D (Minneapolis, MN, USA) kits. Briefly, 5 mL of peripheral blood was collected from each patient in the three groups. After centrifugation at 3500 rpm for 5 min, the supernatant was transferred into a new centrifuge tube and stored in liquid nitrogen. The detection was performed strictly according to the instructions of ELISA kits, with 3 replicates for each sample. The absorbance at 560 nm was determined using the Bio-Rad (Hercules, CA, USA) microplate reader, and converted into the actual concentration of PCT and VEGF based on the standard curves. The mean sensitivity in the test was <0.4 pg/mL, and the inter-batch coefficient of variation was 5.1%.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) software was used for all statistical analysis. Measurement data were expressed as mean ± standard deviation. Inter-group difference was analyzed using analysis of variance, followed by Post-Hoc Test (Least Significant Difference). Correlation analysis was performed using Pearson method. Intestinal flora in liver cancer patients was analyzed using linear discriminant analysis effect size (LEfSe). $p<0.05$ was considered statistically significant.

Results

Changes in Liver Function Indexes and intestinal barrier changes in the Three Groups

As shown in Table I, liver function indexes in peripheral blood of patients with liver cancer changed with the changes in disease condition. With the progression of liver cancer, the level of AST increased significantly, with the highest in advanced liver cancer patients [(91.18±10.34) U/L] ($p=0.046$). However, the level of plasma total protein declined, which was (24.83±1.75) g/L in advanced liver cancer patients ($p=0.035$). Moreover, changes in total bilirubin were significantly associated with the progression of liver cancer ($p=0.003$). Colonoscopy showed that the intestinal barrier of patients in each group had pathological changes of different degrees, and the differences between the groups were not statistically significant.

Changes in Intestinal Flora in the Three Groups

As shown in Figure 1 and 2, the abundance of *Clostridiales*, *Firmicutes*, and *Streptococcus* in the intestinal tract was high in early liver can-

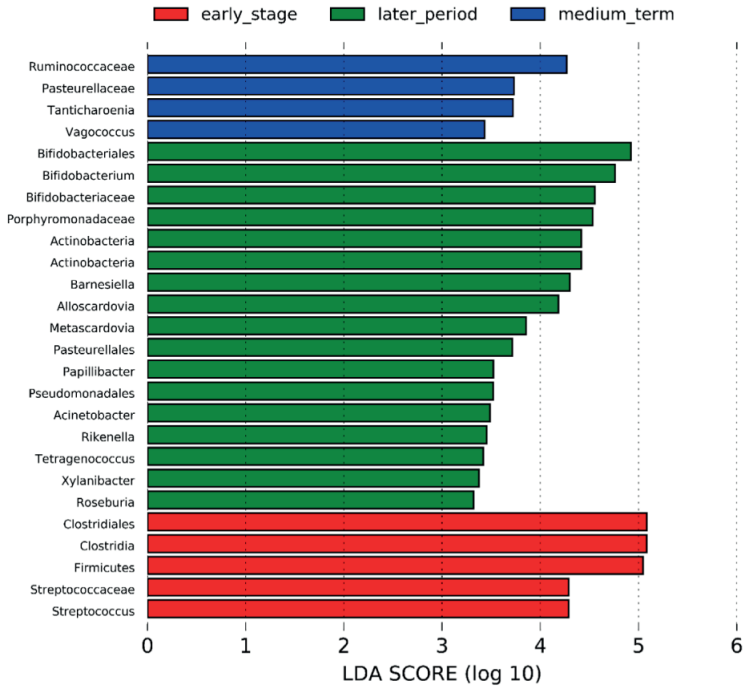

Figure 1. LDA score of intestinal flora in early liver cancer group, middle liver cancer group, and advanced liver cancer group.

cer group. The abundance of *Ruminococcaceae*, *Pasteurellaceae*, *Tanticharoenia*, and *Vagococcus* in the intestinal tract was high in middle liver cancer group. Meanwhile, the abundance of *Bifidobacteriales*, *Actinobacteria*, *Barnesiella*, *Porphyromonadaceae*, and *Pseudomonadales* in the intestinal tract was high in advanced liver cancer group. The levels of *Actinobacteria* (Figure 3) and *Barnesiella* (Figure 4) in advanced liver cancer group were evidently higher than those of the other two groups. However, the level of *Ruminococcaceae* (Figure 5) in advanced liver cancer group was evidently lower than that of the other two groups. Other microorganisms such as Sulfolobaceae did not change significantly between the groups.

Correlation Analysis of Intestinal Flora in the Three Groups

As shown in Figure 6, the level of *Enterobacteriaceae* was positively correlated with that of *Firmicutes* (r=0.36, p=0.003), whereas it was negatively correlated with *Lactobacillus* (r=-0.72, p=0.021). The level of *Lactobacillus* was positively correlated with that of *Ruminococcaceae* (r=0.39, p=0.043), whereas it was negatively correlated with that of *Firmicutes* (r=-0.27, p=0.019).

Changes in Plasma ET and PCT in the Three Groups

The level of PCT significantly rose in advanced liver cancer group [(6.89±0.35) ng/mL] (p=0.021). Furthermore, the level of ET increased remark-

Table I. Changes in liver function indexes in the three groups.

Group	No.	AST (U/L)	ALT (U/L)	TP (g/L)	ALB (g/L)	GGT (U/L)	STB (μmol/L)
Early liver cancer group	100	42.41±2.14	78.48±11.83	62.15±2.14	38.15±1.24	45.15±3.17	21.41±1.84
Middle liver cancer group	100	58.51±3.81	86.11±12.19	65.14±3.81	34.18±2.81	52.87±2.81	25.15±5.18
Advanced liver cancer group	100	91.18±10.34	71.91±18.98	51.35±4.15	24.83±1.75	51.87±1.82	59.85±4.98
F		7.98	2.34	2.59	10.85	1.98	11.75
p		0.046	0.271	0.175	0.035	0.414	0.003

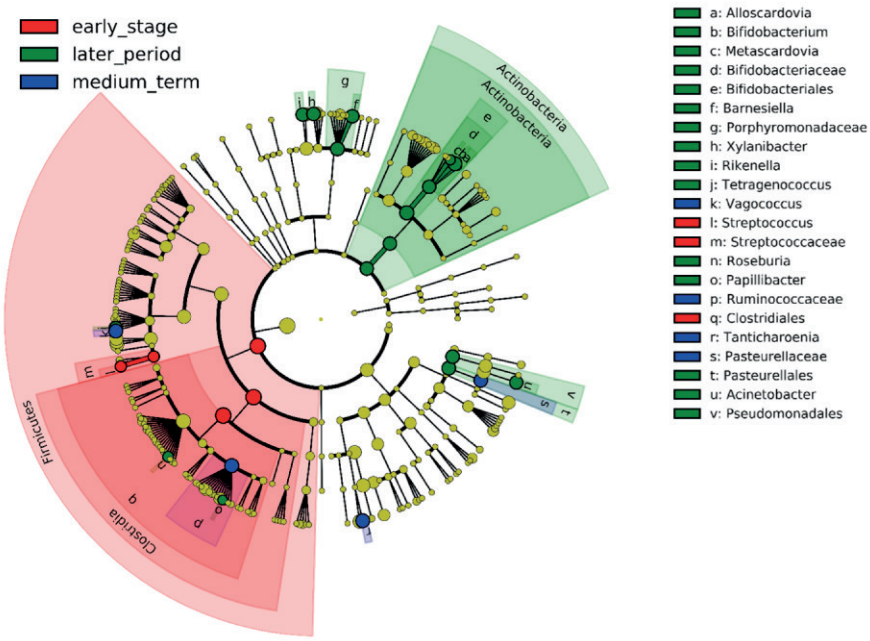

Figure 2. LEfSe of intestinal flora in early liver cancer group, middle liver cancer group, and advanced liver cancer group.

Table II. Changes in liver function indexes in the three groups.

Group	No.	PCT (ng/mL)	ET (EU/mL)
Early liver cancer group	100	3.72±0.76	0.24±0.03
Middle liver cancer group	100	4.24±1.92	0.56±0.11
Advanced liver cancer group	100	6.89±0.35	0.71±0.09
F		17.28	21.87
p		0.021	0.004

ably with the development of liver cancer, with the highest observed in advanced liver cancer group [(0.71±0.09) EU/mL] (p=0.004) (Table II).

Changes in VEGF Level in the Three Groups

VEGF level in liver cancer patients also increased significantly with the aggravation of the disease, which was the highest in advanced liver cancer group [(112.33±2.11) μmol/L], showing differences among groups (p<0.05) (Figure 7).

Discussion

Liver cancer is among the top three diseases in the incidence rate. The occurrence and progression of liver cancer affect the health and life of a large number of people around the world[11,12]. In patients with early liver cancer, the tumor diameter is less than 3 cm without lymph node metastasis or distant metastasis. The treatment means is relatively simple with a better curative effect[13]. In patients with middle-advanced liver cancer, distant spread of tumor stem cells often occurs with blood or lymphatic fluid during tumor growth, making liver cancer prone to relapse or metastasis. Therefore, the efficacy is poor, and the mortality rate is high in these patients[14]. Clearly distinguishing the progression of liver cancer is of great help for the reasonable selection of therapeutic regimen and improvement of therapeutic effect. In this study, liver function indexes in peripheral blood of patients with liver cancer changed with the changes in disease condition. With the progression of liver cancer, the level of AST increased significantly, and the highest was observed in advanced liver cancer patients [(91.18±10.34) U/L] (p=0.046).

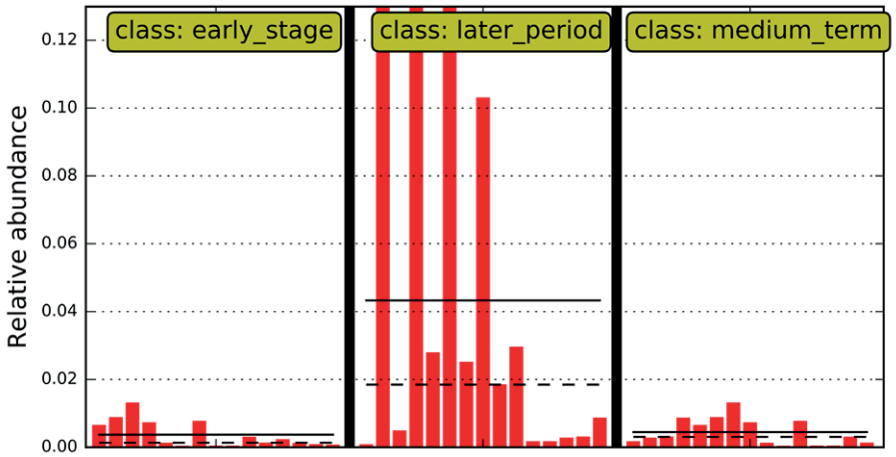

Figure 3. Level of *Actinobacteria* in early liver cancer group, middle liver cancer group, and advanced liver cancer group.

However, the level of plasma total protein declined, which was (24.83±1.75) g/L in advanced liver cancer patients (p=0.035). Changes in total bilirubin were significantly associated with the progression of liver cancer (p=0.003). It could be observed that with the progression of liver cancer, most liver function indexes were gradually dysregulated. Hepatocyte death increased, nutritional status of patients became worse, and jaundice occurred, which was a dynamic process. All these findings confirmed that the progression of liver cancer could lead to the gradual dysregulation or loss of liver function.

Liver cancer is affected by the state and abundance of intestinal microorganisms in patients. Intestinal microorganisms can be used as one of the non-invasive methods for early diagnosis of liver cancer patients[15]. It can also be applied as a potential therapeutic method for liver cancer[16-18]. Intestinal microorganisms have been found related to the occurrence of liver fibrosis after liver cancer[19]. In this study, the abundance of *Clostridiales*, *Firmicutes*, and *Streptococcus* in the intestinal tract was high in early liver cancer group. The abundance of *Ruminococcaceae*, *Pasteurellaceae*, *Tantichaeroenia*, and *Vagococcus* in the intestinal tract was high in middle liver cancer group. The abundance of *Bifidobacteriales*, *Actinobacteria*, *Barnesiella*, *Porphyromonadaceae*, and *Pseudomonadales* in the intestinal tract was high in advanced liver cancer group. Moreover, the levels of *Actinobacteria* and *Barnesiella* in advanced liver cancer group were evidently higher than those of the other two groups. However, the level of *Ruminococcaceae* in advanced liver cancer group was evidently lower than that of the other two groups. The above re-

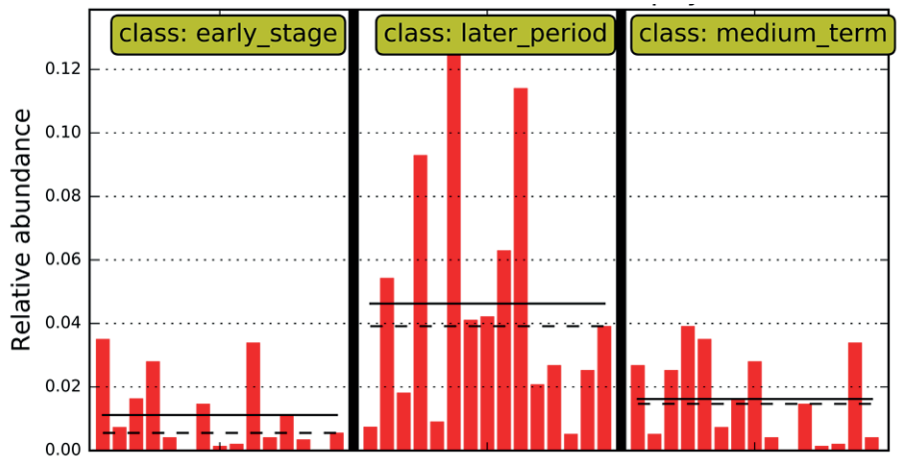

Figure 4. Level of *Barnesiella* in early liver cancer group, middle liver cancer group, and advanced liver cancer group.

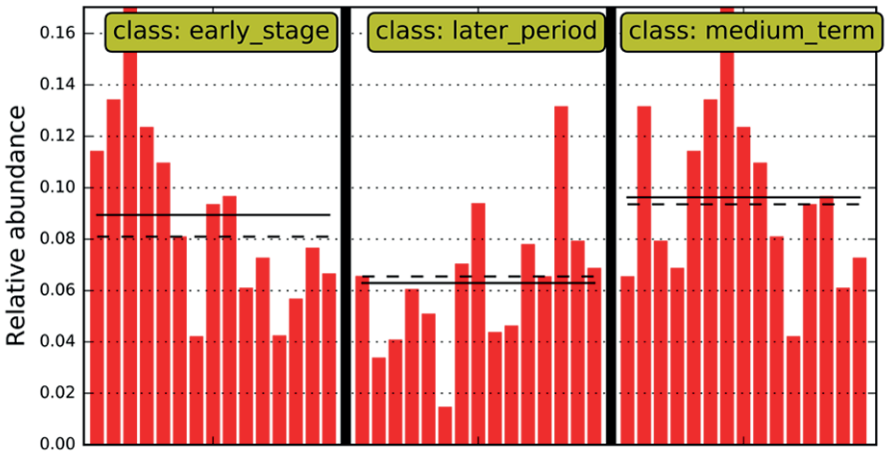

Figure 5. Level of *Ruminococcaceae* in early liver cancer group, middle liver cancer group, and advanced liver cancer group.

sults demonstrated that with the progression of liver cancer, there would be dynamic changes in the composition and level of intestinal microorganisms in liver cancer patients. The possible reason was that intestinal microorganisms were significantly changed to adapt to the disease state of patients. The levels of intestinal probiotics, such as *Ruminococcaceae* and *Firmicutes*, evidently declined in patients with advanced liver cancer. This might be the cause of intestinal dysfunction and nutrient absorption disorder, resulting in further deterioration of liver cancer. In this study, the level of Enterobacteriaceae in patients with liver cancer was positively correlated with that of *Firmicutes* (r=0.36, p=0.003), whereas it was negatively correlated with that of *Lactobacillus* (r=-0.72, p=0.021). Furthermore, the level of *Lactobacillus* was positively correlated with that of *Ruminococcaceae* (r=0.39, p=0.043), but was negatively correlated with *Firmicutes* (r=-0.27, p=0.019). Therefore, it could be speculated that taking *Lactobacillus* preparation might up-regulate the levels of such probiotics as

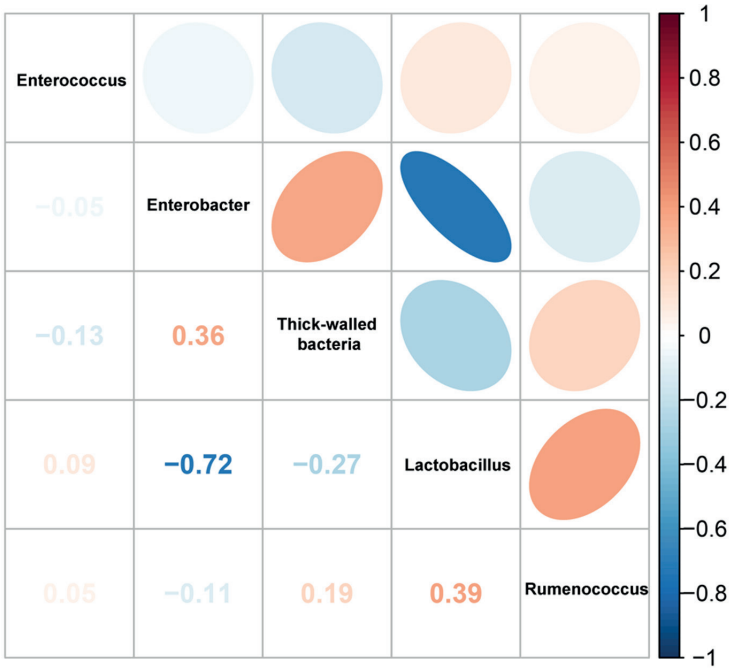

Figure 6. Correlation analysis of intestinal flora in the three groups.

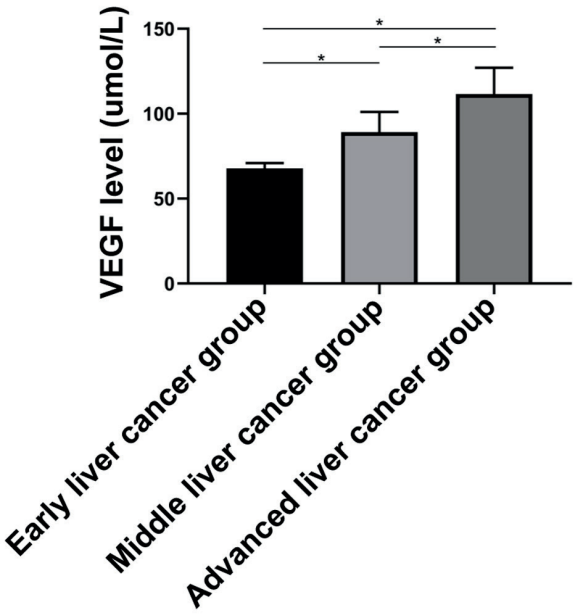

Figure 7. Changes in VEGF level in the three groups (*p<0.05).

Ruminococcaceae in liver cancer patients, improve the intestinal microenvironment, and alleviate the condition of the disease.

PCT and plasma ET often serve as predictors for the presence or absence of infection and its severity[20]. In this study, the level of PCT rose significantly in advanced liver cancer group [(6.89±0.35) ng/mL] (p=0.021). The level of ET increased significantly with the development of liver cancer, which was the highest in advanced liver cancer group [(0.71±0.09) EU/mL] (p=0.004). These results suggested that patients with advanced liver cancer were more susceptible to infection than those with early and middle liver cancer. At the same time, it was found that the level of VEGF in liver cancer patients also increased remarkably with the aggravation of disease, which was the highest in advanced liver cancer group [(112.33±2.11) μmol/L], showing differences among groups (p<0.05).

Conclusions

With the progression of liver cancer, the proliferation rate of tumor cells constantly rises, and vascular permeability also increases, leading to high potential of metastasis. It is also demonstrated that VEGF may be a potential therapeutic target for patients with advanced liver cancer. Furthermore, VEGF inhibitors can be used to prevent tumor proliferation and metastasis, and delay the disease development.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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