Profile of Gut Microbiota Associated With the Presence of Hepatocellular Cancer in Patients With Liver Cirrhosis

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ABSTRACT

Background. Changes within the gut microbiota contribute to the progression of chronic liver diseases. According to the results of several studies performed in animal models, gut dysbiosis plays an important role in hepatocarcinogenesis. The aim of this study was to explore the characteristics of gut microbiota associated with the presence of hepatocellular cancer (HCC) in patients with cirrhosis of the liver undergoing liver transplantation.

Methods. A total of 15 patients with HCC and 15 non-HCC patients matched according to etiology of cirrhosis and Model for End-Stage Liver Disease (MELD) scores who underwent liver transplantations between 2012 and 2014 were included. Analysis of their gut microbial profile was based on prospectively collected stool samples from the pretransplant period.

Results. Patients with and without HCC were similar with respect to age (P = .506), sex (P = .700), hepatitis C virus (P > .999) and hepatitis B virus (P = .715) infection status, alcoholic liver disease (P > .999), and MELD score (P = .337). Notably, the presence of HCC was associated with significantly increased fecal counts of Escherichia coli (P = .025). Prediction of HCC presence based on E coli counts was associated with the area under the receiver-operating curve of 0.742 (95% confidence interval, 0.564–0.920), with the optimal cutoff on the level of 17.728 (natural logarithm of colony-forming units per 1 g of feces). Sensitivity and specificity rates for the established cutoff were 66.7% and 73.3%, respectively.

Conclusions. The profile of gut microbiota associated with the presence of HCC in cirrhotic patients is characterized by increased fecal counts of E coli. Therefore, intestinal overgrowth of E coli may contribute to the process of hepatocarcinogenesis.

THE ROLE of gut microbiota in the development and progression of liver diseases seems substantial [1,2]. Through a system of complex interactions within the gut–liver or microbiota–liver axis, alterations in gut homeostasis may specifically lead to an inflammatory response within the liver and subsequent fibrogenesis [3,4]. Increased production of pro-inflammatory cytokines largely depends on the response of the innate immune system to the presence of microbial products, such as lipopolysaccharides (LPS) or other pathogen-associated molecular patterns recognized by pattern recognition receptors, in the portal circulation [3]. The profile of gut microbiota in patients with liver cirrhosis differs considerably from that observed in patients without liver disease [5–7]. Moreover, the degree of liver insufficiency is closely related to the severity of gut dysbiosis [8].

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Hepatocellular cancer (HCC) is the most frequent primary malignancy of the liver and 1 of the leading causes of cancer-related mortality worldwide [9]. In approximately 80% of patients, the tumors develop in patients with liver cirrhosis. Hepatitis C virus (HCV), excessive alcohol consumption, and hepatitis B virus are the most common underlying etiologic factors [10]. Liver resection and liver transplantation are the only 2 potentially curative therapies available [11]. Although the former is often limited by the presence of cirrhosis, liver transplantation treats both the cancer and the cirrhosis [12]. Thus, HCC is 1 of the most common indications for liver transplantation [13–15]. Notably, inflammation plays an important role in hepatocarcinogenesis [16,17]. Accordingly, inflammatory processes within the liver associated with alterations of gut microbiota might contribute to the development of HCC in patients with liver cirrhosis. Such a hypothesis has been confirmed by findings of several recent studies utilizing animal models of hepatocarcinogenesis [18–21]. Further data on associations between gut microbial characteristics and the occurrence of HCC in humans may facilitate novel methods of identifying patients at higher risk and, possibly, lead to development of novel preventive interventions. Thus, the aim of the present study was to identify associations between the gut microbial profile of patients with liver cirrhosis and the presence of HCC.

PATIENTS AND METHODS

A total of 1554 liver transplantations were performed in the Department of General, Transplant and Liver Surgery at the Medical University of Warsaw (Warsaw, Poland) between 1989 and 2014. After approval of the local ethics committee and provision of informed consent for participation in the study, stool samples were prospectively collected from patients with liver cirrhosis awaiting liver transplantation. Results of microbiologic stool analyses were available for 57 patients who underwent the procedure between 2012 and 2014. The final study cohort comprised 30 patients, including 15 with HCC and 15 without malignancy matched 1:1 based on etiology of cirrhosis and patient laboratory Model for End-Stage Liver Disease (MELD) score. The study was performed in accordance with the Declaration of Helsinki.

Fecal counts of colony-forming units (CFU) per 1 g of wet feces were established for microbiota (Enterococcus species, Escherichia coli, Proteus species, other members of Enterobacteriaceae taxon [Klebsiella, Enterobacter, Citrobacter, and Serratia species], Pseudomonas, Bifidobacterium, Bacteroides, Lactobacillus, and Clostridium species) and yeasts. Details on microbiologic stool analyses have been described in previous reports [7,8]. To characterize the profile of gut microbiota associated with the presence of HCC, results of microbiologic analyses were compared between patients with and without HCC.

Qualitative and quantitative variables are presented as numbers with percentages and medians with ranges, respectively. The Mann-Whitney U test and the Fisher exact test were used to compare groups with respect to qualitative and quantitative variables. Fecal microbial counts were transformed to natural logarithms before analyses. Optimal cutoffs for fecal microbial counts specific for patients with HCC were established based on analyses of the receiver-operating characteristic curves. Areas under the curve are presented with 95% confidence intervals (95% CIs). The level of statistical significance was set at 0.05. STATISTICA version 10 software (StatSoft Inc, Tulsa, Okla, United States) was used for computing statistical analyses.

RESULTS

Of 15 patients with HCC, 14 (93.3%) were within the Milan criteria. The median number of tumors was 1 (range, 1–10); the median size of the largest tumor was 3 cm (range, 1–4.5 cm). Microvascular invasion was present in 2 patients (13.3%). Comparison of baseline characteristics and fecal microbial counts between HCC and non-HCC patients is presented in Table 1. Groups of patients with and without HCC were similar with respect to demographic characteristics (P = .700 for sex and P = .506 for age), etiology of underlying liver cirrhosis (P > .999 for HCV infection and alcoholic liver disease; P = .715 for hepatitis B virus infection), and MELD score (P = .337). Moreover, there were no significant differences between groups regarding particular components of the MELD score, namely serum bilirubin concentration (P = .330), international normalized ratio (P = .177), or serum creatinine concentration (P = .236).

The profile of gut microbiota in patients with HCC was characterized by significantly higher fecal counts of E coli (P = .025) compared with that in non-HCC patients. No

| Table 1. Comparison of Baseline Characteristics and Fecal Microbial Counts Between Patients With and Without HCC |
|-----------------|-----------------|----------------|
|                 | Patients With HCC | Non-HCC Patients |
| Sex             |                 |                  |
| Male            | 9 (60.0)        | 11 (73.3)        |
| Female          | 6 (40.0)        | 4 (26.7)         |
| Age, y          | 59 (27–71)      | 58 (29–67)       |
| Hepatitis C virus infection | 13 (86.7) | 13 (86.7) |
| Alcoholic liver disease | 1 (6.7)  | 1 (6.7) |
| Hepatitis B virus infection | 8 (53.3) | 6 (40.0) |
| MELD            | 10 (6–20)       | 13 (8–18)        |
| Bilirubin       | 1.5 (0.4–5.8)   | 2.0 (0.6–4.4)    |
| INR             | 1.2 (1.0–1.7)   | 1.3 (1.1–1.7)    |
| Creatinine      | 0.7 (0.5–1.3)   | 0.8 (0.6–1.3)    |
| Fecal microbial counts |        |                  |
| Escherichia coli | 18.1 (13.8–21.8) | 16.1 (9.9–20.5) |
| Other           | 9.2 (9.2–18.4)  | 10.6 (9.2–17.7)  |
| Enterobacteriaceae* |        |                  |
| Enterococcus species | 15.9 (9.9–22.5) | 18.1 (9.9–19.8)  |
| Bifidobacterium species | 20.7 (17.5–22.1) | 20.7 (19.1–22.1) |
| Bacteroides species | 21.4 (19.8–22.5) | 21.4 (19.1–22.8) |
| Lactobacillus species | 14.5 (9.8–16.2) | 16.1 (11.5–18.2) |
| H2O2–producing | 12.2 (9.8–18.1) | 16.1 (9.8–18.1)  |
| Lactobacillus species Clostridium species | 9.9 (9.9–15.2) | 9.9 (9.9–13.8) |
| Yeasts          | 6.9 (6.9–12.3)  | 8.5 (6.9–13.1)   |

Data are presented as no. (%) or median (range). Microbial counts are presented as natural logarithm of a number of colony-forming units per 1 g of wet feces.

Abbreviations: H2O2, hydrogen peroxide; HCC, hepatocellular cancer; INR, international normalized ratio; MELD, Model for End-Stage Liver Disease.

*Klebsiella, Enterobacter, Citrobacter, and Serratia species.
significant differences between patients with and without HCC were observed with respect to fecal counts of other \textit{Enterobacteriaceae} \((P = .790)\), \textit{Enterococcus} species \((P = .617)\), \textit{Bifidobacterium} species \((P = .706)\), \textit{Bacteroides} species \((P = .866)\), \textit{Lactobacillus} species in general \((P = .261)\) and those producing hydrogen peroxide \((P = .219)\), \textit{Clostridium} species \((P = .269)\), and yeasts \((P = .386)\).

According to the receiver-operating characteristic curve analysis for fecal count of \textit{E. coli} in prediction of the presence of HCC (Fig 1), the optimal cutoff was 17.728 (natural logarithm of CFU/g). The corresponding area under the curve was 0.742 (95% CI, 0.564–0.920). Sensitivity and specificity rates for the established cutoff were 66.7% and 73.3%, respectively.

**DISCUSSION**

In contrast to increasing number of reports on the associations between gut microbiota and the development of HCC in animal models, limited data derived from studies performed in human subjects are available. In the present study, the profile of gut microbiota associated with the presence of HCC was characterized by considerably increased fecal counts of \textit{E. coli}. Accordingly, increased abundance of \textit{E. coli} in patients with liver cirrhosis may play an important role in the process of hepatocarcinogenesis.

The potential association between increased fecal counts of \textit{E. coli} and the development of HCC is most probably related to the pro-inflammatory effects of LPS within the liver. Using a rat model of diethylnitrosamine (DEN)-induced hepatocarcinogenesis, Zhang et al [18] observed that gut dysbiosis after DEN administration was characterized by a significant elevation in the \textit{E. coli} growth rate, among other changes. Moreover, coadministration of DEN with penicillin aggravated the dysbiotic state with an overgrowth of \textit{E. coli} and undergrowth of the potentially beneficial \textit{Bifidobacterium} and \textit{Lactobacillus} species, increased circulating LPS concentration, and, finally, an increased number and size of tumors. Conversely, high-dose probiotic treatment suppressed the growth of \textit{E. coli} and other gram-negative bacteria and decreased circulating concentrations of LPS. Notably, probiotics not only decreased the number and size of tumors in rats with DEN-induced HCCs but also prevented HCC development in a considerable proportion of rats. Accordingly, the profile of gut microbiota in patients with HCC characterized by \textit{E. coli} overgrowth found in the present study seems to be consistent with the results derived from animal models.

Results of other studies using animal models of hepatocarcinogenesis also point toward the major importance of alterations within the gut microbiota. Dapito et al [21] observed remarkably reduced HCC gross morphologic features in both gut-sterilized and Toll-like receptor-4–mutant mice after administration of DEN and carbon tetrachloride. On the contrary, low-dose LPS infusion had an opposite effect on the induced HCC burden. Accordingly, the results of that previous study provide evidence for the important role of the LPS–Toll-like receptor-4 pathway in hepatocarcinogenesis. In a study by Fox et al [20], colonization of the gut with \textit{Helicobacter hepaticus} considerably aggravated the carcinogenic effect of aflatoxin B1 and promoted carcinogenesis in HCV-transgenic mice. The potential association between \textit{Helicobacter} species and HCC has also been addressed by several studies in humans [22–24].

Given the available evidence derived from previous studies on animal models and the characteristics of the gut microbial profile of patients with HCC found in the present study, restoration of gut homeostasis in patients with chronic liver diseases should be further investigated as a strategy for HCC prevention. Administration of probiotics has been shown to provide several benefits in patients with liver cirrhosis, including improvement of liver function, decreases in hospital admission rates, and recovery of minimal hepatic encephalopathy [25,26]. Most importantly, the results of a recent Phase I study on the treatment with \textit{Lactobacillus} GG of patients with liver cirrhosis and minimal hepatic encephalopathy revealed an efficacy with respect to reducing \textit{Enterobacteriaceae} and the level of endotoxemia [27].

Although a cutoff for fecal \textit{E. coli} count of 17.728 (natural logarithm of CFU/g) was established for predicting the presence of HCC, moderate sensitivity and specificity rates do not seem to be useful for diagnostic purposes. Nevertheless, these rates may be considered helpful for selection of patients who have cirrhosis of the liver and are at higher risk of developing HCC. Accordingly, modulation of microbiota to restore gut homeostasis has the potential to
provide more benefits in cirrhotic patients with fecal E coli counts ≥17.728 (natural logarithm of CFU/g). This possibility, however, needs confirmation in further prospective studies.

Notably, E coli colonization of the colon is being extensively studied as a risk factor for the development of colorectal cancer both in animal-based models and in human subjects [28–30]. However, the effects of increased intestinal abundance of E coli with respect to HCC development seem more related to the circulating LPS rather than direct bacteria–host interactions. Interestingly, E coli–derived LPS has previously been found to induce proliferation of cancer cells more potently than, for instance, Helicobacter pylori–derived LPS [31]. Given that E coli overgrowth and increased circulating concentrations of LPS may contribute not only to development of HCC but also to progression of the malignant disease while patients remain on the waiting list for liver transplantation, this feature might be important in the transplant setting. However, whether such effect exists remains to be elucidated in future studies.

Transplant setting is a major advantage of the present study. Because it was performed in a cohort of liver transplant recipients, no HCC cases were missed because of the rigorous pathologic examination of the explanted livers. However, several limitations of the present study need acknowledgment. First, the number of patients included in this study was relatively small, and, thus, potential associations between components of gut microbial profile other than E coli count might not have been discovered. Moreover, because the study was based on a culture-dependent method of evaluating gut microbiota, the results are limited to the cultured microorganisms. Considering the number of patients with HCC included, there was no possibility of evaluating the associations between HCC features, such as tumor number, size, serum alpha-fetoprotein concentration, microvascular invasion, or tumor differentiation.

CONCLUSIONS

The present study provides evidence for an association between increased fecal counts of E coli and the presence of HCC in cirrhotic livers. Therefore, it seems to confirm the potential role of intestinal overgrowth of E coli and development of HCC observed in animal-based studies. Future studies are necessary to evaluate the efficacy of modulation of gut microbiota as a preventive strategy against HCC development, particularly in patients with high baseline fecal counts of E coli.

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