Early Exposure to Gut Microbiome Reduces Hepatocellular Carcinoma Risks in Mice

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Aims. Liver cancer is a multietiological disease that has multiple factors contributing to the hepatocarcinogenic process, e.g., hepatitis viruses, carcinogens, male sex, or metabolic factors. Notably, emerging evidence reported that gut microbiota is crucial to the pathogenesis of hepatocellular carcinoma (HCC) via activation of innate immunity. However, the effect of time to gut microbiota exposure after birth is unknown. Using a germ-free animal housing environment, instead of antibiotics, we examined the effects of various time-to-exposure (TTE) to gut microbiota durations on HCC risk. Methods. HBV or carcinogen-mediated spontaneous HCC models were implemented in this study. The HCC incidence rates in mice either kept germ-free (GF; that is, with no exposure to gut microbiota) or exposed to gut microbiota after being moved to a specific pathogen-free (SPF) housing environment and with various time-to-exposure (TTE) durations, namely, 5 weeks after birth, 10 weeks after birth, or since conception (that is, 5-week TTE group, 10-week TTE group, and SPF group, respectively), were recorded. The mice were sacrificed at 30 or 40 weeks after birth, and macro-/microscopic observations and pathological diagnosis were performed. Results. The incidence of liver tumors among the male mice was higher than that among the female mice in the carcinogen-induced HCC mice sacrificed at 40 weeks after birth (with $P = 0.011$, $0.035$, 0.0003, and 0.012, respectively, in the GF group, 5-week TTE group, 10-week TTE group, and SPF group). Similarly, in the HBV-HCC model, the incidence of liver tumors among the male mice was significantly higher than that among the female mice (with $P = 0.013$, 0.020, 0.012, and 0.002, respectively, in the GF group, 5-week TTE group, 10-week TTE group, and SPF group). These results suggest that gut microbiota exposure is irrelevant to the male sex preference of HCC. Surprisingly, when comparing carcinogen-induced HCC male mice in the 10-week TTE group (90%; $n = 10$), 5-week TTE group (56%; $n = 9$), and SPF group (30%; $n = 10$) ($P = 0.020$), we found that the incidence of liver tumors was higher in the mice with later exposure to gut microbiota. Similarly, when comparing HBV-HCC male mice in the 10-week TTE group (100%; $n = 11$), 5-week TTE group (70%; $n = 10$), and SPF group (33%; $n = 9$) ($P = 0.080$), we also found that the incidence of liver tumors was higher in the mice with later exposure to gut microbiota. Conclusions. Early (prepubertal) exposure to gut microbiome reduces the risk of HCC development, indicating a potentially important factor for cancer surveillance. Exploring the mechanisms by which such exposure affects HCC risk might lead to novel cancer vaccines.
1. Introduction

Liver cancer is a multietiological disease in which multiple factors, e.g., hepatitis viruses, carcinogens, male sex, and metabolic factors, contribute to the hepatocarcinogenic process. Liver cancer occurs almost exclusively, through a complex process involving hepatocellular injury, inflammation, and compensatory regeneration, in individuals with chronic liver disease. However, the complex mechanisms underlying the disease are still not completely understood. Notably, recent evidence has indicated that the gut microbiota is crucial to the pathogenesis of HCC via the activation of innate immunity. Anatomically, the liver is intimately linked to the gut via the circulatory connection provided by the portal vein. Therefore, the liver is the first responder to microbiota and their products derived from the intestines. In the context of the symbiotic relationship between the host and microbiota, the degree of hepatic exposure to noxious microbes is limited. Once the homeostasis of the microbial ecosystem is disrupted, however, microbe-associated molecular patterns (MAMPs) can evoke inflammatory responses via pattern recognition receptors (PRRs), thereby contributing to chronic liver disease and liver cancer. Indeed, the expression of innate immune receptors has been discovered in multiple types of hepatic cells, including Kupffer cells, hepatocytes, and hepatic stellate cells [1–5], and can effectively provide a surveillance system for recognizing microbial metabolites from the gut. Dapito et al. have shown that gut microbiota promotes the development of HCC via lipopolysaccharide (LPS-) induced Toll-like receptor 4 (TLR4) activation [6]. Yu et al. have revealed that LPS accumulation protects carcinogen-induced apoptosis and promotes liver tumorigenesis in an animal model. Additionally, distinct differences in gut microbiota composition during the development of chronic liver diseases have been demonstrated in a previous animal study [7]. However, the effects of different amounts of time from conception until exposure to gut microbiota are unknown. In this study, therefore, we used a germ-free animal housing environment, instead of antibiotics, to examine the effects of various time-to-exposure (TTE) to gut microbiota durations on HCC risk.

2. Material and Methods

2.1. Mice, HCC Induction, and Evaluation. C57Bl/6 mice were purchased from the National Laboratory Animal Center, and HBV-transgenic mice were provided by Professor Ou of the University of Southern California. The mice received individual intraperitoneal (i.p.) injections of diethyl-nitrosamine (DEN) at ages 10-14 days to induce HCC. Before experiments, the parent mice were gut-sterilized and bred to produce one or two generations of babies to make sure that the mice are absolutely in GF status. Both C57Bl/6 (B6-high DEN) and HBV (HBV-low DEN) mice were randomly divided into four groups (germ-free (GF; no exposure) or various time-to-exposure (TTE; 5 or 10 weeks after birth or exposure ever since conception (that is, 5 wk TTE group, 10 wk TTE group, or SPF group)) and were moved to a specific pathogen-free (SPF) housing environment. Shown are representative images of tumors and H&E sections.
digitally photographed. Furthermore, the number of visible nodules on each liver was counted and measured in term size, after which the given liver was weighed to calculate the liver-to-body weight ratio. The HCC incidence rates of the mice were measured in the four groups (that is, the GF group, 5-week TTE group, 10-week TTE group, and SPF group). Statistics were measured using the chi-squared test and t-test to acquire P value.

2.2. Experimental Design. After the mice were sacrificed, the liver specimens from the 4 groups (that is, the GF group, 5-week TTE group, 10-week TTE group, and SPF group) were analyzed by a professional veterinarian. The liver specimens were examined for macroscopically visible nodules. In addition, microscopic observation and pathological diagnosis with hematoxylin and eosin (H&E) staining were also performed, as shown in Figure 1. The severity of the toxic lesions was graded according to the methods described by Shackelford et al. [8].

3. Results

In order to identify the optimal time of necropsy for our carcinogen-induced HCC model, all of the mice in the four groups (that is, the GF group, 5-week TTE group, 10-week TTE group, and SPF group) were sacrificed at various times (namely, 15 weeks, 20 weeks, 30 weeks, and 40 weeks after birth) at first. No liver tumors were found in the necropsies conducted at 15 weeks after birth, while only a few liver tumors were found in the necropsies conducted at 20 weeks after birth. Far more liver tumors were found in the necropsies conducted at 30 weeks and 40 weeks after birth. Relatedly, the optimal time of necropsy for the HBV-HCC model mice was determined to be 40 weeks after birth. All necropsy results are shown in Table 1.

In the carcinogen-induced HCC model, the incidence of liver tumors among the male mice was higher than that among the female mice sacrificed at 30 weeks after birth (with $P = 0.153$, $0.637$, $0.008$, and $0.582$, respectively, in the GF group, 5-week TTE group, 10-week TTE group, and SPF group), and similar results were found in the mice sacrificed at 40 weeks after birth (with $P = 0.011$, $0.035$, $0.0003$, and $0.012$, respectively, in the GF group, 5-week TTE group, 10-week TTE group, and SPF group). These results are shown in Tables 2 and 3. To confirm the male preference irrelevance to gut microbiome exposure, interaction $P$ values were calculated for the GF group vs. the SPF group and gender. It reported that interaction was $0.398$ in the carcinogen-induced HCC mice sacrificed at 30 weeks after birth and $P$ interaction was $0.816$ in the carcinogen-induced HCC mice sacrificed at 40 weeks after birth. When comparing the GF group (67%; $n = 9$) vs. the SPF group (30%; $n = 10$), we found that the incidence of liver tumors is not significantly different among two groups in male mice ($P = 0.149$). Again, when comparing the GF group (22.2%; $n = 9$) vs. the SPF group (10%; $n = 10$), we found that the incidence of liver tumors is not significantly different among two groups in female mice ($P = 0.582$). However, when comparing the 10-week TTE group (90%; $n = 10$), the 5-week TTE group (56%; $n = 9$), and the SPF group (30%; $n = 10$), we found that the incidence of liver tumors was higher in the male mice with later exposure to gut microbiome ($P = 0.020$) for the necropsies conducted at 30 weeks after birth, as shown in Table 2.

As shown in Table 4, in the HBV-HCC model, the incidence of liver tumors among the male mice was significantly higher than that among the female mice (with $P = 0.013$, $0.020$, $0.012$, and $0.002$, respectively, in the GF group, 5-week TTE group, 10-week TTE group, and SPF group). Again, interaction $P$ values were calculated for the GF group vs. the SPF group and gender. It reported that $P$ interaction was $0.191$ in the HBV-HCC mice sacrificed at 40 weeks after birth. While comparing the GF group (100%; $n = 11$) vs. the SPF group (33%; $n = 9$), we found that the incidence of liver tumor is not significantly different among two groups in male

### Table 1: Liver tumor incidence in carcinogen-induced HCC mice.

| Age | Sex | No. of mice | GF | 10 wk TTE | 5 wk TTE | SPF |
|-----|-----|-------------|----|-----------|----------|-----|
| 15 wks | M | 20 | 0/5 | 0/5 | 0/5 | 0/5 |
|       | F | 25 | 0/6 | 0/6 | 0/7 | 0/6 |
| 20 wks | M | 20 | 1/5 | 1/5 | 1/5 | 0/5 |
|       | F | 24 | 0/6 | 0/6 | 0/7 | 0/5 |
| 30 wks | M | 38 | 6/9 | 9/10 | 5/9 | 3/10 |
|       | F | 38 | 2/9 | 3/11 | 3/8 | 1/10 |
| 40 wks | M | 40 | 6/10 | 7/9 | 4/10 | 6/11 |
|       | F | 43 | 0/10 | 0/12 | 0/11 | 0/10 |

### Table 2: Carcinogen-induced HCC tumor incidence at 30 weeks after birth.

| Variable | GF | 10 wk TTE | 5 wk TTE | SPF | $P^*$ | $P^{**}$ |
|----------|----|-----------|----------|-----|------|--------|
| M       | 6/9 | 9/10 | 5/9 | 3/10 | 0.020 | 0.149 |
| F       | 2/9 | 3/11 | 3/8 | 1/10 | 0.482 | 0.582 |
| $P^{***}$ | 0.153 | 0.0003 | 0.035 | 0.012 |

### Table 3: Carcinogen-induced HCC tumor incidence at 40 weeks after birth.

| Variable | GF | 10 wk TTE | 5 wk TTE | SPF | $P^*$ | $P^{**}$ |
|----------|----|-----------|----------|-----|------|--------|
| M       | 6/10 | 7/9 | 4/10 | 6/11 | 0.307 | 0.8499 |
| F       | 0/10 | 0/12 | 0/11 | 1/10 | 1 | 1 |
| $P^{***}$ | 0.011 | 0.0003 | 0.035 | 0.012 |

### Table 4: HBV-HCC tumor incidence at 40 weeks after birth.

| Variable | GF | 10 wk TTE | 5 wk TTE | SPF | $P^*$ | $P^{**}$ |
|----------|----|-----------|----------|-----|------|--------|
| M       | 7/11 | 11/11 | 7/10 | 3/9 | 0.080 | 0.369 |
| F       | 0/8 | 5/10 | 1/9 | 0/11 | 0.010 | 1 |
| $P^{***}$ | 0.013 | 0.012 | 0.020 | 0.002 |

M: male; F: female. *P value: 10 wk TTE vs. 5 wk TTE vs. SPF. **P value: GF vs. SPF. ***P value: M vs. F.
Furthermore, when comparing the 10-week TTE group (100%; \( n = 11 \)), the 5-week TTE group (70%; \( n = 10 \)), and the SPF group (33%; \( n = 9 \)), we found that the incidence of liver tumors was higher in the male mice with later exposure to gut microbiome (\( P = 0.080 \)). While comparing the 10-week TTE group (50%; \( n = 10 \)), the 5-week TTE group (11%; \( n = 9 \)), and the SPF group (0%; \( n = 11 \)), we found that the incidence of liver tumors was higher in the female mice with later exposure to gut microbiome (\( P = 0.010 \)), as shown in Table 4.

4. Discussion

In the carcinogen-induced HCC model, we found that the liver tumor incidence rate among the male mice was higher than that among the female mice in the necropsies conducted at 30 weeks after birth (with \( P = 0.153, 0.637, 0.008 \), and 0.528, respectively, in the GF group, 5-week TTE group, 10-week TTE group, and SPF group) and at 40 weeks after birth (with \( P = 0.011, 0.035, 0.0003 \), and 0.012, respectively, in the GF group, 5-week TTE group, 10-week TTE group, and SPF group). In the HBV-HCC model, we also found that the liver tumor incidence rate among the male mice was higher than that among the female mice (with \( P = 0.013, 0.020, 0.012 \), and 0.002, respectively, in the GF group, 5-week TTE group, 10-week TTE group, and SPF group). In order to confirm the male preference independently of gut microbiome exposure, interaction \( P \) values were calculated for the GF group vs. the SPF group and gender. It reported the carcinogen-induced HCC mice in the necropsies conducted at 30 weeks after birth (interaction = 0.398) and at 40 weeks after birth (interaction = 0.816), as well as the HBV–HCC mice in the necropsies conducted at 40 weeks after birth (interaction = 0.191). All of these results support the conclusion that gut microbiome exposure is irrelevant to the male preference of HCC. Some other factors involved in the male predilection of HCC need to be investigated.

A comparison of the liver tumor incidence of the GF group vs. the SPF group showed that the liver tumor incidence of the GF group (67%; \( n = 9 \)) was greater than that of the SPF group (30%; \( n = 10 \)) in the male carcinogen-induced HCC mice sacrificed at 30 weeks (\( P = 0.1409 \)); in addition, it was showed that the liver tumor incidence of the GF group (60%; \( n = 10 \)) was greater than that of the SPF group (54.5%; \( n = 11 \)) in the male carcinogen-induced HCC mice sacrificed at 40 weeks (\( P = 0.8499 \)). It was further showed that the liver tumor incidence of the GF group (64%; \( n = 11 \)) was greater than that of the SPF group (33%; \( n = 9 \)) in the HBV–HCC male mice (\( P = 0.369 \)). In contrast, the liver tumor incidence of the GF group (22%; \( n = 9 \)) was greater than that of the SPF group (10%; \( n = 10 \)) in the carcinogen-induced HCC female mice sacrificed at 30 weeks (\( P = 0.582 \)), while there was no difference in the liver tumor incidence of the GF group vs. the SPF group in the female carcinogen-induced HCC and HBV–HCC mice sacrificed at 40 weeks. Though the effect was not significant, the gut microbiota seemed to provide protection from the development of liver tumors in both the carcinogen-induced HCC and HBV–HCC male mice.

To our surprise, a comparison of the 10-week TTE group (90%; \( n = 10 \)), the 5-week TTE group (56%; \( n = 9 \)), and the SPF group (30%; \( n = 10 \)) among the carcinogen-induced HCC male mice (\( P = 0.020 \)), as well as of the 10-week TTE group (100%; \( n = 11 \)), 5-week TTE group (70%; \( n = 10 \)), and SPF group (33%; \( n = 9 \)) among the HBV–HCC male mice (\( P = 0.080 \)), indicated that the incidence of liver tumor was higher in those mice with later exposure to gut microbiome. Similarly, the liver tumor incidence in the 10-week TTE group (50%; \( n = 10 \)) was greater than that in the 5-week TTE group (11%; \( n = 9 \)) and greater than that in the SPF group (0%; \( n = 11 \)) in the HBV–HCC female mice (\( P = 0.010 \)).

Taken together, these results suggest that early exposure to gut microbiome reduces the risk of HCC development. In fact, early exposure to microbiota is suggested to play a critical role in immune development, and, in turn, the immune system shapes the microbial ecology [9]. Studies have shown that innate immune receptors, such as TLRs and Nod-like receptors (NLRs), are important for governing microbiota composition and orchestrating host-microbiota homeostasis in the gut [10–12]. Therefore, a lack of microbial signaling might result in innate immunity deficits and change the composition of gut microbiota [13], which might potentially cause bacterial dysbiosis and gut barrier dysfunction [14]. For example, previous studies of GF mice have revealed that the commensal microbiota regulates the expression of genes engaged in several important intestinal functions [14] and the host immune system in maintaining tolerance and in reducing the entry of gut bacteria into portal circulation [15]. Furthermore, our findings may be explained by the “hygiene hypothesis” [16], which suggests that inadequate microbial exposure during early life may incline an individual to uncontrolled inflammation-associated diseases in later life [17–19]. In addition, early life exposures to various microorganisms encourage a protective TREG phenotype that suppresses cancer [17]. In contrast, hygienic individuals with few exposures in earlier life may experience a dysregulated TREG feedback loop leading to an increased risk of malignancy later in life [17].

Current knowledge regarding the gut-liver axis focuses on leaky gut, microbial dysbiosis, pathogen-associated molecular patterns (PAMPs), and bacterial metabolites [2]. The treatment of some GF mouse models with cocktails of antibiotics has revealed that bacterial dysbiosis at the late survival times to microbiota. Our GF mice had earlier gut sterilization (since birth) compared to Dapito et al.’s or Ma et al.’s
GF mice (who were sterilized at age of 2 weeks or 5 weeks) [6, 20]. The phenomenon of early exposure to gut microbiome may modulate the immune system and healthy microbiota [19]. Studies with other animal models also have shown that the depletion of microbiota during early life results in increases in the number of invariant natural killer T (iNKT) cells and a predisposition to the development of inflammatory diseases. All of the dynamic complex reciprocal interactions between microbiota and HCC through MAMPs, PRRs, bile acid, the farnesoid X-activated receptor- (FXR-) dependent and epidermal growth factor receptor- (EGFR-) dependent pathway, and protein chemokine (C-X-C motif) ligand16 (CXCL16) support our hypothesis of an “endocrine organ-like tumor” (EOLT) [22–28], in which crosstalk occurs between a tumor and its host in an endocrine manner [27]. More studies need to be conducted, however, to further elucidate the relationship between microbiome and cancer and thus further illuminate the EOLT hypothesis [28].

As noted, the tumor incidence in the resource of HBV-transgenic mice is more than 80% [29], which is higher than this report (as in Table 4, HBV-HCC, male, SPF, about 33%). While comparing the experimental conditions, we used the same wtTg08 transgenic mouse as the creator [29]. The HBVtg-HCC mice received DEN (2 mg/kg body weight) injection at 16 days of age, while the same dosing of DEN was injected at ages 10–14 days. Despite the minor difference on HCC-inducing procedure, there are different times, area, and facilities between the literature and this report. We speculated that a distinctive SPF (specific pathogen-free) examination checklist among various institutions might cause data discrepancy. Such data discrepancy is consistent with the concept that microbiota alters severity or incidence of HCC in mice.

5. Conclusions

Early exposure to gut microbiome reduces the risk of HCC development, indicating a potentially important factor for cancer surveillance. A better understanding of early microbial signaling could potentially allow for the development of a cancer vaccine to prevent HCC in the future.

Data Availability

All datasets are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors have declared that no competing interests exist.

Authors’ Contributions

YM Lee executed and drafted the manuscript; FJ Lei helped with animal experiments; CT Kor, WC Chang, and HC Lai analyzed the data; YL Chen and WL Ma initiated and supported the project and final approval of the manuscript.

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