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Article

Relationship of Metabolic Dysfunction-Associated Steatohepatitis-Related Hepatocellular Carcinoma with Oral and Intestinal Microbiota: A Cross-Sectional Pilot Study

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Abstract: Background: The incidence of metabolic dysfunction-associated steatohepatitis (MASH)-related hepatocellular carcinoma (HCC) is increasing annually with a rise in the incidence of obesity and metabolic syndrome. Based on preliminary reports regarding the potential association of HCC and periodontitis, this study aimed to analyze the involvement of periodontal bacteria as well as the oral and intestinal bacterial flora in MASH-related HCC (MASH-HCC). Methods: Forty-one patients with MASH and 19 with MASH-HCC who completed survey questionnaires, underwent periodontal examinations, and subjected to sample collection (saliva, mouth-rinsed water, feces, and peripheral blood) participated in the study. Bayesian network analysis was used to analyze the causation between various factors, including MASH-HCC, examinations, and bacteria. Results: The occupancy rate of the genus *Fusobacterium* in the intestinal bacterial flora was significantly higher in in the MASH-HCC group than in the MASH group ($p = 0.002$), whereas that of *Butyricicoccus* ($p = 0.022$) and *Roseburia* ($p < 0.05$) was significantly lower. Bayesian network analysis revealed the absence of periodontal pathogenic bacteria and enteric bacteria affecting HCC. However, HCC directly affected the periodontal bacterial species *Porphyromonas gingivalis*, *Tannerella forsythia*, *Fusobacterium nucleatum*, and *Prevotella intermedia* in the saliva, as well as the genera *Lactobacillus*, *Roseburia*, *Fusobacterium*, *Prevotella*, *Clostridium*, *Ruminococcus*, *Trabulsiella*, and SMB53 in the intestine. Furthermore, *P. gingivalis* in the oral cavity directly affected the genera *Lactobacillus* and *Streptococcus* in the intestine. Conclusion: MASH-HCC directly affects periodontal pathogenic

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and intestinal bacteria, and *P. gingivalis* may affect the intestinal bacteria associated with gastrointestinal cancer.

Keywords: microbiota; oral bacteria; intestinal bacteria; hepatocellular cancer; metabolic dysfunction-associated steatohepatitis; *Porphyromonas gingivalis*

1. Introduction

Hepatocellular carcinoma (HCC) evolves from chronic liver disease and accounts for 85% of all primary liver cancers [1]. Viral hepatitis was the primary cause, whereas advances in treatment have gradually lowered its incidence [2]. Meanwhile, the incidence of HCC (MASH-HCC) related to metabolic dysfunction-associated steatohepatitis (MASH, formerly known as NASH) [3] has increased over the years owing to an increase in the incidence of obesity and metabolic syndrome [4]. However, the pathogenesis of MASH-HCC remains unclear.

To date, the mechanism by which periodontal bacteria affect the entire body is hypothesized to be as follows: bacteria enter the bloodstream directly from periodontal pocket ulceration and affect organs outside the oral cavity [5,6]. In recent studies, another mechanism has been identified: oral bacteria in saliva are transferred enterally to the intestine by swallowing and affect the intestinal bacterial flora and metabolism [7,8]. The liver is an organ of the digestive system that is anatomically and physiologically connected to the enterohepatic circulation via the portal vein. Hence, periodontal bacteria and lipopolysaccharides (LPS) derived from periodontal bacteria in the saliva may be implicated in the pathogenic mechanism of MASH-HCC by affecting the intestinal flora.

In recent years, epidemiological reports have shown that periodontal pathogen is a risk factor in onset of various cancers and cancer-associated mortality [9]. *Fusobacterium nucleatum* was specifically detected in organs of the digestive system that are anatomically close to the oral cavity [10–12], and the presence of *Porphyromonas gingivalis* was correlated with the malignancy of esophageal cancer [13]. Only a few reports have shown an association between HCC and periodontal pathogen, such as high circulating reactive oxygen species levels in patients with HCC and periodontitis [14]. As far as we are aware, our recent report showing an association between MASH-HCC and salivary *P. gingivalis*, *F. nucleatum*, and IgA is the only report to date highlighting a relationship between MASH-HCC and periodontopathic bacteria [15].

Based on these reports, we hypothesized that MASH-HCC is associated with periodontopathic bacteria in the oral cavity. This study aimed to analyze the clinical parameters and oral and intestinal bacterial flora in patients with MASH and MASH-HCC to determine the relationship between MASH-HCC and periodontal bacteria.

2. Materials and Methods

2.1. Participants

Participants in this study included patients with MASH and MASH-HCC aged 20 years or older who attended or were admitted to the Department of Gastroenterology at Yokohama City University (YCU) Hospital between November 2020 and April 2022. Those who were taking antimicrobials within one month prior to periodontal examination and those with edentulous jaws were excluded from the study. This study was approved by the research ethics committee of Kanagawa Dental University (KDU) and YCU and was conducted at YCU Hospital in compliance with the Declaration of Helsinki. All participants were informed of the purpose, outline, safety, and protection of personal information of this study, and their written consent to participate in the study based on their free will was obtained. Initially, sixty-nine participants were enrolled, and data from sixty participants (forty-one with MASH and nineteen with MASH-HCC) for whom all testing and sample collection data were available were used for the analysis.

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2.2. Background Information

The participants' gender, age and smoking status were interviewed using a questionnaire form. The dentist filled out the response form based on the participants' responses. Body mass index (BMI) was calculated from the medical records by obtaining the height and weight values closest to the date of periodontal examination.

2.3. Periodontal Examination

Periodontal examinations were performed by two dentists from the Department of Periodontology at KDU. The probing depth and bleeding on probing were measured at the six probing points per tooth. The plaque index was recorded at the four points per tooth, and tooth mobility was evaluated. Probing was performed at constant pressure using a plastic probe (Contact Probe, Nihon Dental Laboratory Co., Ltd., Tokyo, Japan) with a probing pressure of 0.2 N. The dentists calibrated their probing tools in advance. A periodontal jaw model (P15FE-500HPRO-S2A1-GSF, Nissin, Kyoto, Japan) was used for calibration.

2.4. Sample Collection

Saliva samples used for IgA concentration assay were collected using the SALIVET® (SARSTEDT, Nümbrecht, Germany). A polypropylene-polyethylene polymer sponge was held under the tongue for 2 min, and the saliva-containing sponge was returned to the tube. The tubes were quickly ice-cooled, centrifuged (1,200 × g, 20 min, 4 °C), and stored at –80 °C until analysis. Mouth-rinsed water was used to analyze the oral microbiota. Participants rinsed with saline for 10 s and then collected into tubes. The rinsed water was stored at –80 °C until analysis. Fecal samples were collected from the participants using a Mykinso fecal collection kit® (Cykinso, Inc., Tokyo, Japan) containing a guanidine thiocyanate solution. Fecal samples were collected by the participants themselves according to the manufacturer's manual.

2.5. Medical Examination

Peripheral blood samples were collected on the same day as periodontal examination. Endotoxin, high-sensitivity C-reactive protein (CRP), aspartate transaminase (AST), alanine aminotransferase (ALT), and total bilirubin (T-Bil) levels were analyzed.

2.6. Assay of IgA Concentration in Saliva

IgA concentration in saliva were determined by enzyme-linked immunosorbent assay (ELISA) using a Human IgA ELISA Kit (Bethyl Laboratories, Inc., Montgomery, TX, USA). The ELISA was performed according to the manufacturer's instructions.

2.7. DNA Preparation and Microbiota Analysis

DNA extraction and bacterial flora analysis of the mouth-rinsed water and fecal samples were performed at the Medical Laboratory (Cykinso, Inc.) [16,17]. DNA was extracted using an automated DNA extraction machine (GENE PREP STAR PI-1200A; Kurabo Industries Ltd., Osaka, Japan) according to the manufacturer's protocol. Detailed sequencing methods are described in previous report [18]. Data processing and assignment were performed using the QIIME2 pipeline (version 2020.8), and based on the work of Fujihara et al. [19].

2.8. Bayesian Network Analysis and Classification Trees

A Bayesian network is a directed acyclic graph composed of a set of variables {X1, X2,...,XN} and a set of directed edges between them [20]. The details of the analytical methods are described in our previous report [21]. Because the Bayesian network could not be analyzed with missing values, we excluded one participant from the MASH group who had missing T-Bil values, and data from fifty-nine participants (forty patients with MASH and nineteen patients with MASH-HCC) were used for

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the analysis. Based on the results of Bayesian network analysis, a classification tree analysis was performed using rpart.

2.9. Statistical Analysis

Statistical analyses were performed using SPSS Statistics (version 27.0; IBM, Tokyo, Japan) and R (version 3.5.1 (The R Project for Statistical Computing, Vienna, Austria, 2018). The Mann–Whitney's U test was used for comparisons between two groups, except for gender and smoking status, which were verified by χ^2 test. The Spearman's rank correlation coefficient was used for the correlation analysis. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Participant's Information, Periodontal and Medical Examinations, and Salivary IgA Levels

Table 1 shows information on the participants, periodontal and medical status, and IgA concentration in saliva (the data is also reported in our previous study [15]). Compared with the MASH group, the MASH-HCC group was significantly older ($p = 0.0004$). Both groups showed similar periodontal examination results. The salivary IgA concentration was significantly lower in the MASH-HCC group than in the MASH group ($p < 0.001$). Endotoxin and T-Bil levels were significantly higher in the MASH-HCC group ($p < 0.0001$) than in the MASH group ($p = 0.014$).

Table 1. Demographic factors, periodontal and medical conditions, and salivary IgA levels.

Parameter	MASH Group (N = 41)	MASH-HCC Group (N = 19)	*p*-Value
Gender (men/women)	25/16	13/6	0.578
Smoking status (+/−)	9/32	4/15	0.973
Age (years)	59 (55–70)	79 (64–82)	0.0004*
BMI	26.2 (22.3–31.5)	27.7 (25.9–30.8)	0.503
Number of remaining teeth	26 (21–27)	25 (21–27)	0.598
PD (mm)	2.8 (2.6–3.2)	2.9 (2.6–3.2)	0.653
BOP (%)	14.9 (10.7–24.1)	15.4 (7.3–31.4)	0.799
Tooth mobility	0 (0–0.1)	0 (0–0.1)	0.703
PlI	0.9 (0.8–1.4)	0.9 (0.7–1.4)	0.619
Salivary IgA (ug/mL)	231.7 (146.5–482.8)	102.7 (85.8–168.9)	<0.001*
Endotoxin (EU)	0.13 (0.08–0.17)	0.22 (0.15–0.28)	<0.0001*
CRP (mg/dL)	0.14 (0.09–0.48)	0.13 (0.07–0.34)	0.487
AST (U/L)	51 (27–62)	38 (29–58)	0.546
ALT (U/L)	53 (26–71)	29 (23–44)	0.094
T-Bil (mg/dL)	0.8 (0.6–1)	1.3 (0.7–1.7)	0.014*

BMI, body mass index; PD, Probing depth; BOP, Bleeding on probing; PlI, Plaque index; CRP, C-reactive protein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; T-Bil, total bilirubin. BOP (+) = 1; BOP (−) = 0. The figures for gender and smoking status indicate the number of individuals, and statistical analysis was performed by χ^2 test. The presented values for the other items are medians (first quartile–third quartile), and the Mann-Whitney's U test was used for statistical analysis (*$p < 0.05$).

3.2. Diversity and Composition of Bacterial Flora in the Saliva

Table 2 shows the diversity and abundance of the salivary microbiome. All bacterial phyla detected, 23 bacterial genera with an average occupancy greater than 0.5%, and 22 bacterial species with an average occupancy greater than 0.1% are presented. The Shannon index of the salivary microflora was significantly lower in the MASH-HCC group than in the MASH group ($p = 0.03$). Regardless of liver disease type, the predominant microorganisms at the phylum level in all participants were *Bacillota*, *Bacteroidota*, *Pseudomonadota*, *Actinomycetota* and *Fusobacteriota*. Similarly,

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the genera *Streptococcus*, *Prevotella*, *Veillonella* and *Actinomyces* predominated in both groups. Their occupancy rate was not significantly different between the two groups.

The proportions of *P. gingivalis* and *F. nucleatum* were higher in the MASH-HCC group than in the MASH group. However, only *F. nucleatum* showed a significant difference ($p = 0.014$). Conversely, the occupancy rate of *Treponema denticola* was significantly lower in the MASH-HCC group than in the MASH group ($p = 0.02$).

Table 2. Bacterial phyla, genera and species of the salivary microbiota.

Parameter	MASH Group (N = 41)	MASH-HCC Group (N = 19)	*p*-Value
Diversity of bacterial flora			
Shannon index	6.72 (6.45–7.13)	6.51 (6.05–6.80)	0.03*
Phylum			
Actinomycetota (%)	11.7 (9.56–15.19)	13.15 (10.38–14.25)	0.661
Bacillota (%)	39.5 (36.94–43.06)	42.7 (35.56–45.30)	0.604
Bacteroidota (%)	24.94 (21.61–28.02)	23.45 (20.54–30.08)	0.450
Campylobacterota (%)	0.976 (0.618–1.50)	1.13(0.806–1.57)	0.418
Cyanobacteria (%)	0 (0–0)	0 (0–0)	0.928
Desulfobacterota (%)	0 (0–0.0346)	0.0135 (0–0.0208)	0.945
Fusobacteriota (%)	8.12 (3.94–9.81)	8.23 (5.42–9.61)	0.335
Patescibacteria (%)	1.83 (0.463–2.54)	1.32 (0.337–2.01)	0.167
Pseudomonadota (%)	13.93 (6.79–16.71)	13.17 (3.01–17.39)	0.727
Spirochaetota (%)	0.269 (0.025–0.638)	0.061 (0.029–0.386)	0.185
Synergistetes (%)	0.040 (0–0.103)	0.020 (0.013–0.025)	0.228
Genus			
Actinomyces (%)	6.27 (3.62–8.05)	5.39 (3.84–6.72)	0.418
Alloprevotella (%)	1.54 (0.788–3.26)	1.16 (0.691–1.91)	0.348
Atopobium (%)	0.337 (0.165–0.774)	0.495 (0.147–0.768)	0.962
Campylobacter (%)	1.02 (0.650–1.50)	1.13 (0.806–1.57)	0.525
Capnocytophaga (%)	0.897 (0.282–1.85)	0.976 (0.524–1.70)	0.391
Corynebacterium (%)	0.306 (0.191–0.804)	0.484 (0.146–0.931)	0.340
Fusobacterium (%)	3.25 (1.84–5.07)	4.48 (2.24–5.58)	0.266
Gemella (%)	2.15 (1.14–3.07)	2.04 (1.17–3.29)	0.949
Granulicatella (%)	2.73 (1.86–3.73)	2.39 (1.60–3.16)	0.221
Haemophilus (%)	3.36 (1.70–5.34)	3.96 (1.10–5.77)	0.836
Lactobacillus (%)	0.00620 (0–0.239)	0.122 (0–1.02)	0.247
Lautropia (%)	0.141 (0–0.633)	0.138 (0–0.831)	0.572
Leptotrichia (%)	2.83 (1.36–4.58)	2.23 (1.53–3.84)	0.567
Megasphaera (%)	0.413 (0.151–0.873)	0.536 (0.106–1.03)	0.861
Neisseria (%)	5.20 (1.14–8.72)	1.25 (1.03–9.20)	0.505
Peptostreptococcus (%)	0.256 (0.0694–0.643)	0.183 (0–0.664)	0.262
Porphyromonas (%)	3.71 (1.72–7.14)	3.98 (0.900–6.49)	0.589
Prevotella (%)	16.1 (11.8–19.3)	15.9 (11.6–20.6)	0.799
Rothia (%)	3.09 (1.53–5.53)	4.42 (2.38–5.42)	0.409
Streptococcus (%)	20.3 (16.1–24.6)	22.2 (16.0–27.0)	0.340
TM7x (%)	0.729 (0.221–1.509)	0.463 (0.0127–1.04)	0.249
Treponema (%)	0.292 (0.0331–0.561)	0.0611 (0.0302–0.225)	0.0768
Veillonella (%)	7.98 (6.07–10.7)	9.68 (6.30–12.4)	0.204
Species			
Actinomyces israelii (%)	0 (0–0)	0 (0–0.0256)	0.101
Bifidobacterium dentium (%)	0 (0–0.040)	0 (0–0.0748)	0.620
Capnocytophaga gingivalis (%)	0.345 (0.130–0.691)	0.427 (0.154–1.25)	0.193

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Dialister pneumosintes (%)	0 (0–0.0821)	0 (0–0.0587)	0.917
Fusobacterium nucleatum (%)	0.189 (0.0200–0.443)	0.362 (0.170–0.928)	0.014*
Lactobacillus crispatus (%)	0 (0–0)	0 (0–0.00302)	0.544
Lactobacillus fermentum (%)	0 (0–0.287)	0 (0–0.150)	0.661
Lactobacillus gasseri (%)	0 (0–0.0257)	0 (0–0.122)	0.165
Lactobacillus salivarius (%)	0 (0–0.00405)	0 (0–0.0874)	0.363
Metamycoplasma hyosynoviae (%)	0 (0–0.0576)	0 (0–0.0599)	1.000
Porphyromonas endodontalis (%)	0.457(0.138–0.982)	0.195(0–0.377)	0.0923
Porphyromonas gingivalis (%)	0.138 (0–1.26)	0.400 (0–0.725)	0.520
Prevotella denticola (%)	0.459 (0.0750–0.948)	0.429 (0.0683–0.924)	0.905
Prevotella enoeca (%)	0 (0–0.00780)	0 (0–0)	0.149
Prevotella intermedia (%)	0 (0–0.334)	0 (0–0.158)	0.532
Prevotella nigrescens (%)	0 (0–0.0243)	0 (0–0)	0.175
Streptococcus anginosus (%)	0.124 (0.0379–0.247)	0.165 (0.0126–0.373)	0.937
Streptococcus mutans (%)	0.0110 (0–0.0608)	0.0685 (0–0.118)	0.159
Streptococcus pneumoniae (%)	0.557 (0.277–0.998)	0.376(0–1.43)	0.260
Streptococcus sobrinus (%)	0 (0–0.0183)	0(0–0.00838)	0.919
Tannerella forsythia (%)	0.146 (0.0612–0.287)	0.0827 (0.0200–0.328)	0.661
Treponema denticola (%)	0.0638 (0–0.195)	0 (0–0.0325)	0.0223*

Values are presented as medians (first quartile–third quartile), and the Mann-Whitney's U test was used for statistical analysis (*$p < 0.05$).

3.3. Diversity and Composition of Bacterial Flora in the Feces

Table 3 shows the diversity and abundance of bacterial flora in feces, including all bacterial phyla detected and 23 bacterial genera with top occupancy. The Shannon index was significantly lower in the MASH-HCC group than in the MASH group ($p < 0.001$). At the phylum level, both groups were dominated by *Bacillota*, *Bacteroidota*, *Actinomycetota*, and *Pseudomonadota*. However, occupancy rates were comparable between the two groups. Only *Fusobacteriota* was significantly more prevalent in the MASH-HCC group than in the MASH group ($p = 0.002$).

At the genus level, *Bacteroides* and *Blautia* dominated all participants, but their occupancy rates were comparable between the two groups. The occupancy rates of *Butyricicoccus* ($p = 0.022$) and *Roseburia* ($p < 0.05$) in the MASH-HCC group were significantly lower than those in the MASH group. Conversely, the occupancy rate of *Fusobacterium* in the MASH-HCC group was significantly higher than in the MASH group ($p = 0.002$).

Table 3. Bacterial phyla and genera of the fecal microbiota.

Parameter	MASH Group (N = 41)	MASH-HCC Group (N = 19)	*p*-Value
Diversity of bacterial flora			
Shannon index	5.79 (5.54–6.17)	5.43 (4.92–5.56)	<0.001*
Phylum			
Actinomycetota (%)	5.92 (2.54–9.96)	4.62 (1.91–9.57)	0.515
Bacillota (%)	46.8 (42.1–51.1)	45.9 (42.1–51.7)	0.793
Bacteroidota (%)	37.3(31.5-42.6)	35.7(31.2-41.2)	0.634
Campylobacterota (%)	0(0-0)	0(0-0)	0.307
Desulfobacterota (%)	0.210(0.0138-0.687)	0.203(0-0.753)	0.719
Fusobacteriota (%)	0.0172 (0–0.452)	1.11 (0.263–2.32)	0.002*
Patescibacteria (%)	0(0-0)	0(0-0)	0.580
Pseudomonadota (%)	4.98 (3.99–10.6)	6.11 (3.46–12.3)	0.861
Spirochaetota (%)	0 (0–0)	0 (0–0)	0.197
Synergistetes (%)	0 (0–0)	0 (0–0)	1.000
Verrucomicrobia (%)	0 (0–0)	0 (0–0)	0.776

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Genus			
Bacteroides (%)	29.8 (19.0–34.5)	25.6 (19.4–29.4)	0.360
Bifidobacterium (%)	3.84 (0.39–9.48)	1.87 (1.02–7.72)	0.836
Blautia (%)	7.16 (4.66–10.8)	8.11 (1.31–10.4)	0.424
Butyricicoccus (%)	0.540 (0.195–0.825)	0.170 (0.029–0.370)	0.022*
Clostridium (%)	0.310 (0.165–0.820)	0.270 (0.130–0.770)	0.594
Collinsella (%)	1.83 (0.0100–2.98)	1.20 (0.0100–3.00)	0.930
Coprococcus (%)	1.51 (0.375–4.17)	0.830 (0.140–2.00)	0.133
Dorea (%)	2.70 (1.71–4.62)	2.68 (0.460–4.05)	0.259
Faecalibacterium (%)	1.68 (0–6.16)	0.119 (0–3.45)	0.282
Fusobacterium (%)	0.0100 (0–0.550)	1.11 (0.260–2.00)	0.002*
Lachnospira (%)	0.479 (0.0150–1.65)	0.070 (0.0100–1.00)	0.292
Lactobacillus (%)	0.0200 (0–0.505)	0.220 (0–8.54)	0.104
Oscillospira (%)	0.610 (0.205–1.43)	1.19 (0.290–1.75)	0.500
Parabacteroides (%)	2.51 (1.26–5.93)	3.42 (1.73–5.83)	0.490
Prevotella (%)	0.0100 (0–0.0300)	0.0200 (0.0100–0.850)	0.074
Roseburia (%)	0.970 (0.380–1.76)	0.360 (0.150–1.29)	<0.05*
Ruminococcus (%)	1.38 (0.105–4.00)	0.239 (0.110–1.80)	0.294
Serratia (%)	0.0200 (0–0.560)	0.0100 (0–4.36)	0.562
SMB53 (%)	0.110 (0.0200–0.265)	0.239 (0–0.650)	0.707
Streptococcus (%)	0.790 (0.190–3.45)	1.56 (0.210–4.42)	0.634
Sutterella (%)	3.20 (0.985–4.12)	2.13 (0.580–3.87)	0.259
Trabulsiella (%)	0.270 (0.040–0.920)	0.440 (0.090–2.39)	0.499
Veillonela (%)	0.0500 (0–1.23)	0.050 (0.0100–0.650)	0.797

Values are presented as medians (first quartile–third quartile), and the Mann-Whitney's U test was used for statistical analysis (* $p < 0.05$).

3.4. Determination of Causal Effects Using Bayesian Network Analysis

Figure 1 shows the results of the Bayesian network analysis. We focused on the items that showed significant differences in the comparison between the two groups (Tables 1–3) and added the major oral periodontopathic bacterial species and the top 23 enterobacterial genera (Table 3), setting them as factors in the Bayesian network analysis.

The presence of HCC directly affected the following major periodontal bacterial species: *P. gingivalis*, *Tannerella forsythia*, *F. nucleatum*, and *Prevotella intermedia* in the saliva. It also affects salivary IgA concentrations. Furthermore, salivary IgA concentrations affect *Prevotella intermedia* in saliva.

HCC also directly affected the genera Lactobacillus, Roseburia, Fusobacterium, Prevotella, Clostridium, Ruminococcus, Trabulsiella, and SMB53 in the feces. In addition, P. gingivalis in the saliva directly affected Lactobacillus and Streptococcus in the feces and indirectly affected Blautia and Butyricicoccus. Moreover, salivary F. nucleatum affected Serratia in feces. Meanwhile, T-Bil level and age had a direct impact on HCC. The genus Oscillospira in the feces affected T-Bil.

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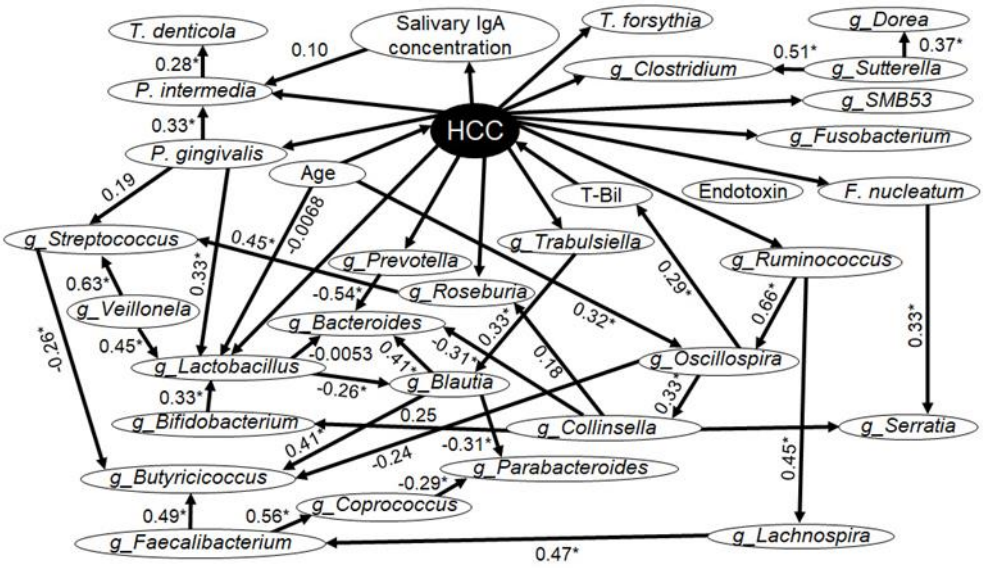

Figure 1. Bayesian network with the graphical representation of causal relationships between factors. The analyzed factors consisted of a total of 32 items (4 items that showed significant differences in Table 1, 5 oral periodontal bacterial species, and 23 genera of top occupying intestinal bacteria). g_: enterobacterial genera, HCC: hepatocellular carcinoma. The source of the arrow is the cause and the destination is the effect. The numbers listed on the side of the arrows are Spearman's rank correlation coefficients (n = 59). Statistical superiority was defined as $p < 0.05$, in which case the numbers were marked with an *.

3.5. Classification Tree to Assess Disease Type

The Bayesian network results showed that the two factors affecting HCC were T-Bil level and age. When the dependent variable was set as the presence or absence of HCC and the explanatory variables were set as T-Bil and age in a classification tree (Figure 2), the major factor affecting HCC was T-Bil, followed by age. HCC develops when T-Bil exceeds 1.35 mg/dl in the MASH. However, even if the T-Bil is less than 1.35 in MASH, HCC develops when the patient is over 77 years of age.

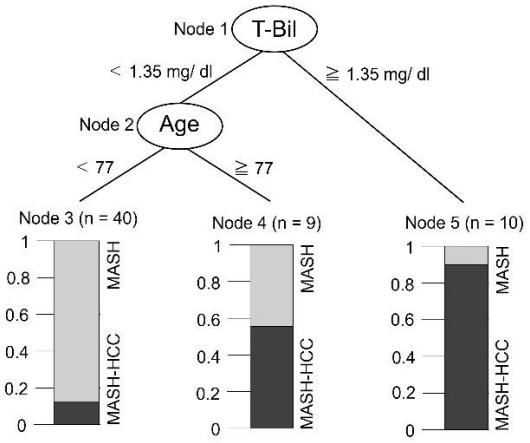

Figure 2. Validated classification tree with hepatocellular carcinoma (HCC) as the dependent variable and total bilirubin (T-Bil) and age as the explanatory variables.

4. Discussion

This is the first study to analyze the relationship between oral periodontal pathogenic bacteria and intestinal bacteria in patients with MASH and MASH-HCC. In this study, the presence of HCC directly affected several periodontopathogenic bacteria in the saliva. In addition, a higher abundance

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of *F. nucleatum* in saliva was observed in the MASH-HCC group than in the MASH group. It has been reported that oral bacterial flora changes in patients with pancreatic cancer [22], and the presence of *F. nucleatum* in the oral cavity is elevated in patients with lung and colorectal cancers [23,24]. Cancer occurs when the systemic immune response decreases [25], and immune function clearly decreases in patients with cancer [26]. In addition, since the type and number of oral bacteria are related to systemic immune status [27], in this study, the decline in systemic immune function caused by MASH-HCC may have affected periodontal bacteria in the oral cavity, which is a remote organ.

The abundance of salivary *F. nucleatum* in the MASH-HCC group was higher than that of other periodontal pathogenic bacteria, except for *P. gingivalis*. *F. nucleatum* is an opportunistic bacterium present in the oral cavity of individuals without periodontal disease [28]. In addition, Leigh et al. [29] reported that opportunistic bacteria in the oral cavity increase owing to a decline in immune function. Hence, although there was no difference in periodontal conditions between the two groups in this study, the MASH-HCC group had decreased systemic immune function; therefore, the occupancy rate of *F. nucleatum* in the oral cavity may have been high.

Bayesian network analysis revealed that HCC directly affected several fecal bacteria; however, none of the fecal bacteria directly affected HCC. Among the intestinal bacteria directly affected by HCC, the genus *Roseburia* was found to have a lower abundance in the MASH-HCC group than in the MASH group, whereas the genus *Fusobacterium* had a higher abundance. Various studies have reported that gastrointestinal cancer is associated with the intestinal microbiome. The genus *Roseburia* was decreased in the intestinal microbiota of patients with colorectal and pancreatic cancer [30,31]. Although there is a known case of MASH that developed into MASH-HCC through liver cirrhosis [32], *Roseburia* occupancy was decreased in the intestinal microbiota of patients with liver cirrhosis [33]. The abundance of the genus *Fusobacterium* increases in the feces of patients with colorectal cancer [34,35]. The abundance of *Fusobacterium* increases, and that of *Roseburia* decreases when dysbiosis occurs in the intestinal microbiota [36]. The liver and intestinal microbiomes have a close bidirectional relationship [37], and dysbiosis occurs in patients with liver cancer [38]. Moreover, the Shannon Index, which shows the diversity of the intestinal microflora, decrease owing to dysbiosis [39]. The Shannon index was lower in the MASH-HCC group than in the MASH group in this study. Hence, it is likely that the MASH-HCC group in the present study had more advanced dysbiosis.

High-fat, high-glucose, and low-fiber Western diets are known to accelerate progression from MASH to MASH-HCC [40]. In addition, the Western diet increases the abundance of the genus *Fusobacterium* and decreases the abundance of the genus *Roseburia* in the intestine [41]. Thus, high-fat, high-glucose, and low-fiber diets that cause HCC may also affect the intestinal bacteria.

Our results showed that the genus *Fusobacterium* in feces did not affect HCC, and *F. nucleatum* in the saliva did not affect the genus *Fusobacterium* in feces. Guo et al. reported that *F. nucleatum* is increased in hepatocellular carcinoma tissues and that hepatocellular carcinoma is affected by *F. nucleatum* because methyltransferase-like protein 3 expression during *F. nucleatum* infection is involved in tumor progression [42]. Although not revealed in the present study, intestinal bacteria may affect HCC.

Primary bile acids increase in MASH-HCC, and *Lactobacillus*, which metabolizes them, has been reported to increase in the intestine [43]. Therefore, Bayesian network analysis revealed a direct effect of MASH-HCC on *Lactobacillus* spp in feces. Interestingly, not only HCC but also *P. gingivalis* in the saliva directly affected the genus *Lactobacillus* in feces. *P. gingivalis* is a typical periodontal pathogenic bacterium in the oral cavity that can affect intestinal bacteria and cause dysbiosis [44]. Park et al. [45] found that mice infected with *P. gingivalis* in the oral cavity showed increased levels of the intestinal phyla *Actinobacteria* and *Deferribacteres*. In addition, Nakajima et al. [46] reported that a single oral dose of *P. gingivalis* administered to mice increased *Bacteroidetes* and decreased *Firmicutes* in the intestine. Oral administration of *P. gingivalis* causes changes in the intestinal microbiota, impairs intestinal barrier function, and damages the liver [46]. Although the difference was not significant, the occupancy rate of salivary *P. gingivalis* was higher in the MASH-HCC group. Hence, in addition to HCC, the high abundance of *P. gingivalis* in saliva could have damaged the liver and altered the amount of primary bile acids, which could have affected the genus *Lactobacillus*.

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We found that *P. gingivalis* in the saliva had a direct effect on the genus *Streptococcus* in the feces. In patients with atrophic gastritis in the gastric corpus who are at a high risk of gastric cancer, an increase in *Streptococcus* spp. in the stomach [47] and in the feces of patients with CRC [48] has been reported, and the genus *Streptococcus* is associated with digestive disorders. Thus, in the MASH-HCC group, an increase in *P. gingivalis* in the oral cavity can cause dysbiosis in the intestine, which may have affected *Streptococcus* spp.

The causal analysis indicated that salivary *P. gingivalis* caused a decrease in *Blautia* and *Bacteroides* via the genus *Lactobacillus* and *Butyricicoccus* via the genus *Streptococcus*. Studies have shown an association between these three intestinal bacteria and gastrointestinal cancer. *Blautia* spp. are decreased in the feces of liver cancer patients [49], *Bacteroides* spp. are decreased in the feces of mice that developed liver cancer due to a high-fat, high-cholesterol diet [50], and *Butyricicoccus* spp. are decreased in the feces of patients with esophageal cancer [51]. All of these bacterial genera are short-chain fatty acid (SCFA) producers [52–54]. McBrearty et al. [55] reported that since SCFAs have strong anti-inflammatory and antitumor effects, the administration of SCFA to mice delayed the development of hepatocellular carcinoma. These bacterial genera did not directly affect HCC but may have affected HCC via SCFA. Hence, reducing *P. gingivalis* in the oral cavity, which indirectly affects these three intestinal bacteria, may help prevent the development of MASH-HCC.

Our data demonstrated that salivary *F. nucleatum* affected the fecal *Serratia* spp., which is an opportunistic bacterium like *F. nucleatum* [27,56]. Lin et al. [57] reported increased levels of both oral *F. nucleatum* and gut opportunistic bacteria of a mouse model of ulcerative colitis. In the present study, patients with MASH-HCC would have had a generalized state of weakened immune system that made them susceptible to an increase in both the opportunistic bacteria *F. nucleatum* and the genus *Serratia*. Therefore, our results show that *F. nucleatum* in the oral cavity directly affects *Serratia.*

Interestingly, salivary IgA concentrations only affected *P. intermedia* in the saliva. Salivary IgA levels increase with the number of periodontal pathogenic bacteria and control them [58–60]. Despite this, the fact that salivary IgA concentration only affected *P. intermedia* in this study suggests that the effect of HCC on periodontal pathogenic bacteria in the oral cavity was greater than that of the salivary IgA concentration.

Of the two factors directly affecting HCC, one was blood T-Bil level, which was directly affected by fecal *Oscillospira* spp. T-Bil levels increase as liver function declines in patients with liver cancer [61]. Increased T-Bil levels have also been reported in rats with liver cancer [62]. Therefore, it is likely that the MASH-HCC group in this study showed a decline in liver function, resulting in high T-Bil levels. Furthermore, an increase in secondary bile acids produced by intestinal bacteria decreases liver function [63], and the level of secondary bile acids in feces is positively correlated with genus *Oscillospira* in feces [64]. The genus *Oscillospira* may have affected the increase in T-Bil levels by reducing liver function through secondary bile acids.

Age is another factor that directly affects HCC development. The median age of the patients in the MASH-HCC group was higher than that in the MASH group. Recently, Shimomura et al. [65] reported that patients with MASH-HCC were older and had lower antioxidant function than patients with MASH and that oxidative stress correlated with MASH activation markers, both of which were increased. However, young patients had lower levels of MASH activation markers because their antioxidant functions were preserved [65]. Hence, old age may be a major risk factor for MASH development.

The results of the classification tree analysis suggested that a T-Bil of 1.35 mg/ dL or higher in the MASH group was related to the occurrence of HCC. HCC occurrences at the age of 77 years or older, even when T-Bil is less than 1.35 mg/dL in MASH. Therefore, MASH patients with T-Bil of 1.35 mg/dL or higher, or older MASH patients with T-Bil less than 1.35 mg/dL, may require more medical assistance to prevent them from developing HCC. High T-Bil levels are the major factors affecting HCC. The genus *Oscillospira*, which elevates T-Bil levels, increases when dysbiosis occurs in the intestines [66]. Because *P. gingivalis* in the oral cavity causes intestinal dysbiosis [46], patients with MASH may require periodontal management to suppress the abundance of *P. gingivalis* in the oral cavity to prevent dysbiosis.

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Limitations

The current study has several limitations. First, the number of participants was low. This is because it was difficult to recruit participants who met the inclusion criteria. Therefore, the number of participants in the two groups could not be matched. Second, differences were observed in the ages of participants in the MASH and MASH-HCC groups. Future studies will need to set the age of the participants higher in order to keep the age of both groups the same.

5. Conclusions

MASH-HCC directly affects periodontal pathogenic bacteria, salivary IgA, and intestinal bacteria. *P. gingivalis* may, directly and indirectly, affect the intestinal bacteria associated with gastrointestinal cancer.

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