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ORIGINAL ARTICLE

Association of gut microbiome and primary liver cancer: A two-sample Mendelian randomization and case–control study

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Abstract

Background and Aims: Observational epidemiology studies suggested a relationship between the gut microbiome and primary liver cancer. However, the causal relationship remains unclear because of confounding factors and reverse causality. We aimed to explore the causal role of the gut microbiome in the development of primary liver cancer, including hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC).

Methods: Mendelian randomization (MR) study was conducted using summary statistics from genome-wide association studies (GWAS) of the gut microbiome and liver cancer, and sequencing data from a case–control study validated the findings. A 5-cohort GWAS study in Germany (N = 8956) served as exposure, whilst the UK biobank GWAS study (N = 456 348) served as an outcome. The case–control study was conducted at the First Affiliated Hospital of Wenzhou Medical University from December 2018 to October 2020 and included 184 HCC patients, 63 ICC patients and 40 healthy controls.

Results: A total of 57 features were available for MR analysis, and protective causal associations were identified for *Family_Ruminococcaceae* (OR = 0.46 [95% CI, 0.26–0.82]; p = .009) and *Genus_Porphyromonadaceae* (OR = 0.59 [95% CI, 0.42–0.83]; p = .003) with HCC, and for *Family_Porphyromonadaceae* (OR = 0.36 [95% CI, 0.14–0.94]; p = .036) and *Genus_Bacteroidetes* (OR = 0.55 [95% CI, 0.34–0.90]; p = .017) with ICC respectively. The case–control study results showed that the healthy controls had a higher relative abundance of *Family_Ruminococcaceae* (p = .00033), *Family_Porphyromonadaceae* (p = .0055) and *Genus_Bacteroidetes* (p = .021) than the liver cancer patients.

Conclusions: This study demonstrates that *Ruminococcaceae*, *Porphyromonadaceae* and *Bacteroidetes* are related to a reduced risk of liver cancer (HCC or ICC), suggesting potential significance for the prevention and control of liver cancer.

Abbreviations: BMI, body mass index; AFP, alpha-fetoprotein; CA19-9, carbohydrate antigen 19–9; CCA, cholangiocarcinoma; CEA, carcinoembryonic antigen; EAF, effect allele frequency; GWAS, genome-wide association studies; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; IV, instrumental variables; MR, Mendelian randomization; RA, relative abundance; IVW, inverse-variance weighted; SNP, single nucleotide polymorphisms.

KEYWORDS
case–control study, gut microbiome, hepatocellular carcinoma, intrahepatic cholangiocarcinoma, Mendelian randomization

1 | INTRODUCTION

Primary liver cancer is the most frequent fatal malignancy globally. It causes at least 788 000 deaths per year currently,[1] as well as the 5th and 16th significant causes of death in the USA and worldwide respectively.[1,2] The World Health Organization (WHO) estimates that the death of liver cancer patients will increase yearly, exceeding one million by 2030.[3] Hepatocellular carcinoma (HCC) is the predominant type of primary liver cancer,[4] accounting for approximately 75% of the total liver cancer cases globally.[5] HCC has the highest incidence in eastern Asia,[6] whilst China has the most significant number of patients because of its large population (1.4 billion persons) and elevated incidence(18.3 per 100 000).[5] Cholangiocarcinoma (CCA) is the second leading primary liver cancer following HCC.[7] In particular, intrahepatic cholangiocarcinoma (ICC) is a highly heterogeneous primary epithelial carcinoma of the liver.[8,9] Over the past 10 to 20 years, the incidence and mortality of ICC have shown a steady upward trend.[9] As liver cancers are usually diagnosed at an advanced stage and exacerbate the global disease burden, it is urgent to find the cause and take appropriate preventive measures to address the increasing number of incidences year by year.

The gut microbiome, a complicated combination of multiple microorganisms, is critical in controlling human health and diseases.[10] Microbial composition imbalance and dysfunction can cause various diseases once an individual's gut microbiome is in metabolic disorder.[11] The gut microbiome can be regarded as a new 'organ', which contains more than hundreds of genes than the host and is affected by genetic and environmental factors.[12] Research has revealed that the gut microbiome is linked to various diseases, including obesity,[13] diabetes,[14] immune diseases,[15] cardiovascular disorders[16] and neurological diseases.[15,17]

The gut-liver axis is an interactive pathway connecting the gut (including the gut microbiome) and the liver[18]; endogenous and exogenous substrates in the gut and liver are translocated through the portal vein and bile duct.[19] Alcohol abuse and obesity would increase intestinal permeability, and the immune system can recognize the relevant microorganisms and microbial products after translocation, causing an inflammatory cascade response.[19,20] The liver and the gut are intricately linked physiologically.[19] The liver serves as one of the targets of the gut microbiome; poor gut status can lead to chronic inflammation and affect the process of liver diseases, eventually leading to liver cancer.[21] Even though there were some observational epidemiology studies that revealed the relationship between the gut microbiome and liver cancer.[22,23] However, it is difficult to confirm the causality between the gut microbiome and liver cancer through observational epidemiology studies because of potential confounding factors and reverse causality.

Lay Summary

Observational epidemiology studies suggested a relationship between the gut microbiome and primary liver cancer. However, the causal relationship remains unclear because of confounding factors and reverse causality. This study revealed the causal relationship between the gut microbiome and primary liver cancer by Mendelian randomization (MR). The results suggested that *Ruminococcaceae*, *Porphyromonadaceae* and *Bacteroidetes* had a protective causal association with primary liver cancer. In addition, these findings of MR were validated by 16s rRNA sequencing data by constructing a case–control study.

In this study, we used a two-sample Mendelian randomization (MR) method to investigate the role of the gut microbiome in the development of HCC and ICC. Furthermore, we validated the relationship by sequencing data from a case–control study. We aimed to explore the causality between the gut microbiome and liver cancer, to contribute to the theoretical basis for the aetiology of liver cancer in order to prevent the occurrence of liver cancer.

2 | METHODS

2.1 | Mendelian randomization

2.1.1 | Assumptions of MR

MR is a novel statistical method focusing on genetic variation that uses single nucleotide polymorphisms (SNPs) as instrumental variables (IVs) to control confounding factors.[24] MR depends on the random assignment of genetic variants during meiosis,[25] implying that confounding factors can be overcome and provide a superior advantage in exposure-outcome causal inference since genotypes were determined before birth.[26] MR uses summary statistics from genome-wide association studies (GWAS) of large amounts of genetic variation to extract SNPs associated with exposure and outcome variables, further revealing clear causal relationships between the exposure and outcome.[27] In this study, SNP effect estimates in GWAS summary statistics related to gut microbiome and liver cancer risk served as exposure and outcome respectively. The MR approach predates three main assumptions (Figure 1). First, SNPs used as IVs significantly correlate with gut microbiome and reach the genome-wide significance threshold (Assumption 1). Second, IVs are independent of confounding factors (Assumption 2). Third, the IVs should only affect liver cancer through

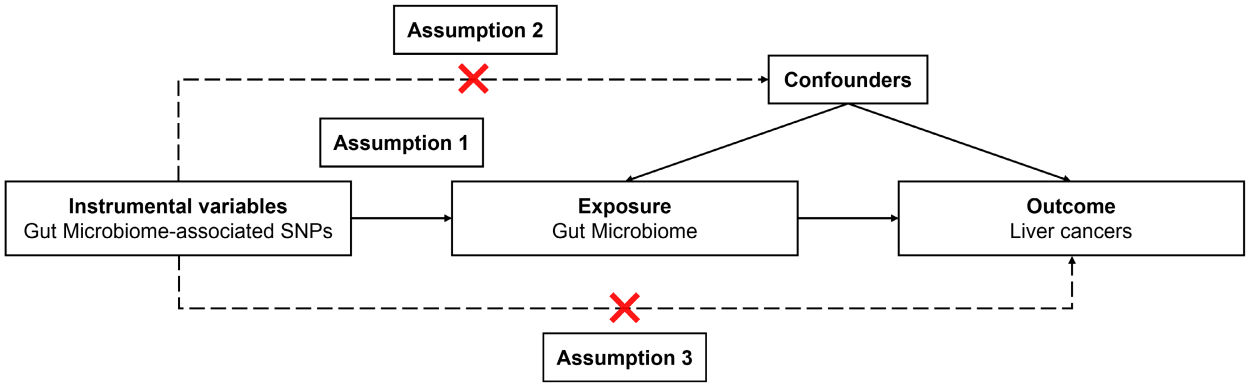

FIGURE 1 Three main assumptions of Mendelian randomization.

the gut microbiome, and other pathways or routes are unavailable (Assumption 3).

2.1.2 | GWAS summary statistics

All analyses for MR in this study were based on summary statistics from GWAS studies of the gut microbiome, HCC and ICC. The data were obtained from previously published studies, and GWAS summary statistics for the gut microbiome, HCC and ICC are available for download from the GWAS catalogue (https://www.ebi.ac.uk/gwas/). Therefore, no review agency approval was required.

Summary statistics from a gut microbiome GWAS study, including 8956 German individuals, served as exposure data.[28] The GWAS study comprised five independent cohorts from northern Germany, and appropriate quality control revealed similarities in baseline information, genomic variation and gut microbiome amongst the cohorts. This GWAS study met the second MR assumption that genetic variants were unconfounded by comparing anthropometric measures (age, body mass index [BMI], gender), genetic variation and microbial community compositions amongst cohorts. Ultimately, the GWAS study identified 44 significant genome-wide associations related to gut microbiome characteristics and community composition, involving 38 genomic loci.

Summary statistics for HCC and ICC obtained from UK Biobank (containing 456348 individuals, 11842647 variants and 2989 binary traits) GWAS study,[29] HCC and ICC were strictly defined by the International Classification of Diseases Tenth Revision (ICD-10) codes. The exposure data were from the German population, whilst the outcome data were from the UK population. Populations from two independent samples of the same ethnic background can overcome the winner's curse bias caused by sample overlap.[30]

2.1.3 | IV selection

We extracted all data provided in the GWAS summary statistics of the gut microbiome, where traits of phylum, class, order, family and genus were marked with increasing relative abundance (RA).[28] As a result of the small number of SNPs reaching the genome-wide statistical significance threshold ($p < 5 \times 10^{-8}$) for each level of the gut microbiome, we adjusted the threshold to the locus-wide significance level ($p < 1 \times 10^{-5}$) to obtain complete and reliable results after referring to the relevant literature. In parallel, we performed quality control according to the following steps: First, we used the PLINK clumping method with a more stringent clumping threshold ($r^2 < 0.001$, $kb = 10000$) to ensure that SNPs in LD within a particular window were pruned to assess the bias caused by residual LD of genetic variants.[31] Second, the *F*-statistic is a critical index for MR to evaluate whether weak IVs are confounded in the selected IVs.[32] We calculated the *F*-statistic for all included SNPs, and in general, an *F*-statistic >10 could overcome weak IVs bias. Third, when SNPs associated with exposure were not present in the outcome data, we performed subsequent analyses by finding and selecting appropriate proxy SNPs ($r^2 > 0.8$). Fourth, cirrhosis, hepatitis B virus (HBV) infection and non-alcoholic fatty liver disease (NAFLD) were risk factors for primary liver cancer and potentially influenced MR findings as confounding factors. We screened two commonly used databases containing publicly available information on a large number of genetic variants, the PhenoScanner[33] (http://www.phenoscanner.medschl.cam.ac.uk/) database and the Phenome-wide association study[34] (PheWAS, https://gwas.mrcieu.ac.uk/phewas/) database, to identify and remove genetic variants used as IVs directly associated with cirrhosis, HBV infection and NAFLD to address the MR assumptions. Fifth, SNPs with a palindromic structure were excluded automatically during the analysis.

2.1.4 | MR analysis

Three main methods, including inverse-variance weighted (IVW), Egger regression and weighted median, were applied for the two-sample MR analysis to estimate the causal relationship between the gut microbiome and HCC or ICC respectively. The IVW method is a meta-analysis of multiple Wald ratios ($\beta_{MR} = \beta_{outcome} / \beta_{exposure}$) weighted by the square of the standard error (SE) outcome variable.[35] The Wald ratio is the final causal association assessment when only one SNP is available, although the result's credibility will be

significantly reduced. Egger regression differs from IVW by adding an intercept term for detecting horizontal pleiotropy, the MR-Egger intercept test.[35,36] The weighted median method allows SNPs with a more extensive beta to contribute more to the estimation.[31] The advantage of the weighted median method is that only half of the valid SNPs can provide unbiased causal estimates.[31]

In addition, we performed a leave-one-out sensitivity test to explore if a single SNP dominated the inference of causal associations. Cochran's *Q* statistics were also performed to confirm the heterogeneity between the selected IVs.[37] MR pleiotropy residual sum and outlier (MR-PRESSO) test and Radial MR were supplemented to detect outliers that potentially cause horizontal pleiotropy.[38] MR robust adjusted profile score (MR-RAPS) can robust inference for MR analysis with weak IV, especially when exposure and outcome are complex traits.[39]

The above analyses were performed with the *TwoSampleMR* package (V 0.5.6), *mr. raps* (V 0.2), *Radial MR* (V 1.0) and *MR-PRESSO* (V 1.0) in the R program (R Foundation for Statistical Computing, V 4.2.0). $p < .05$ was considered statistically significant. In particular, the *TwoSampleMR* package integrated summary statistics from numerous exposure and outcome GWAS studies, including the following information: SNP ID, chromosome, position, beta (β), SE, effect allele (EA), other allele (OA), effect allele frequency (EAF, if available) and *p*-value.

2.2 | Case–control study

2.2.1 | Participant recruitment

The case–control study was conducted at the First Affiliated Hospital of Wenzhou Medical University from Dec.2018 to Oct.2020. HCC patients and ICC patients were selected from the Department of Hepatobiliary Surgery and were diagnosed by CT scan or MRI and confirmed by biopsy of the tumour tissue after surgery. The exclusion criteria for patients were as listed below: (1) ≤18 years old; (2) with other malignant tumours history; (3) with other gastrointestinal disorders; (4) received antibiotic or probiotic treatment within the last 2 months prior to surgery; (5) participants with incomplete information. Healthy controls were selected simultaneously from the Physical Examination Center of the First Affiliated Hospital of Wenzhou Medical University. The exclusion criteria for healthy controls were as follows: (1) ≤18 years old; (2) with liver disorders, gastrointestinal disorders, or other tumours; (3) with chronic non-communicable diseases; (4) with incomplete information. Faecal samples were routinely collected from all healthy controls, HCC patients and ICC patients before treatment to avoid the effects of treatment or surgery on the features and composition of the patients' gut microbiome. Simultaneously, baseline demographic information of all participants and the clinical characteristics of HCC patients and ICC patients were collected. It includes gender, age, BMI, smoking history, drinking history, HBV infection history, cirrhosis history, alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA) and carbohydrate antigen 19–9 (CA19-9). Finally, 287 participants met the inclusion criteria, including 40 healthy controls, 184 HCC patients and 63 ICC patients. This study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University (Ref No. 2020–074) and all participants signed an informed consent form.

2.2.2 | Faecal sample storage, DNA extraction and 16s rRNA sequencing

All participants' faecal samples were frozen and stored in a −80°C refrigerator after collection immediately. In order to reduce the effect of collecting samples with different faecal locations, we collected the middle faeces of all participants as samples. The genomic DNA of the gut microbiome was extracted by the EZNA® Stool DNA Kit (D4015, Omega, Inc.). The DNA extraction process was performed using ultra-pure water to avoid false-positive PCR results. High-throughput V3-V4 regions of 16s rRNA identified gut microbiome classification and composition in faecal samples. Subsequently, the amplification of the V3-V4 high-throughput region of 16s rRNA via PCR reaction (Thermo Scientific® Phusion High-Fidelity Hot start flex 2X Master Mix [New England Biolabs]) with primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′). The PCR products were purified with AMPure XT beads (Beckman Coulter Genomics) and quantified with Qubit (Invitrogen, USA). The amplicon pools were prepared for sequencing by the Agilent 2100 Bioanalyzer (Agilent). The Library Quantification Kit for Illumina (Kapa Biosciences) estimated the size and number of amplicon libraries. Samples were sequenced on the Illumina NovaSeq platform, and LC-Bio Technologies Ltd provided sequencing support.

2.2.3 | Bioinformatics analysis and statistical analysis

The raw reads were analysed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) of the QIIME2 software. Quality filtering of raw reads is implemented by fqtrim software (V 0.94). DADA2 filtered the sequencing reads and output feature tags and sequences. Owing to the accuracy of the algorithm, fewer spurious sequences could be output. Species annotation was completed by BLAST, databases for comparison using SLIVA and NT-16 S. Alpha-diversity was described by the Chao1 index, Shannon index and Simpson index, with the *P*-value using the Wilcoxon test. β-diversity was performed by principal coordinates analysis (PCoA) based on weighted UniFrac distance metric and non-metric multidimensional scaling (NMDS) analysis using R *ade4* (V 1.7–18) and *vegan* (V 2.5–7) package. The comparison of the relative abundance of relevant gut microorganisms between HCC patients (or ICC patients) and healthy controls was analysed using the Mann–Whitney test. All data analyses were

conducted by the R program (R Foundation for Statistical Computing, V 4.2.0). $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Mendelian randomization

3.1.1 | IV selection

The flowchart of MR analysis is shown in Figure 2. Initially, 3226 SNPs associated with gut microbiota features ($p < 1 \times 10^{-5}$) were selected as IVs, including five classification levels: phylum, class, order, family and genus. After performing the PLINK clumping process for linkage disequilibrium, 758 SNPs were left for the next analysis. The gut microbiota features of a lower classification level are probably affiliated with a higher classification level, implying that SNPs may partially overlap. Thus, of the gut microbiomes that were causally associated with HCC (*Family_Ruminococcaceae* and *Genus_Porphyromonadaceae*) and ICC (*Family_Porphyromonadaceae* and *Genus_Bacteroidetes*), we calculated the F-statistic with the median 22.12 (*Family_Ruminococcaceae*), 20.35 (*Genus_Porphyromonadaceae*), 21.08 (*Family_Porphyromonadaceae*), 20.52 (*Genus_Bacteroidetes*) respectively. The *F*-statistics were all >10, indicating weak IV bias could be effectively avoided. Two new sets of data integrating relevant SNPs for exposure and outcome were obtained after the 758 SNPs were harmonized with the summary statistics from GWAS studies of HCC and ICC. All IVs were not associated with cirrhosis, HBV infection and NAFLD after the verification from the PhenoScanner and PheWAS databases, implying the MR assumptions were met. The search for proxy SNPs and the removal of palindromic SNPs were completed simultaneously.

3.1.2 | Two-sample MR analysis

After excluding SNPs because of linkage disequilibrium and palindrome structure, 14 to 21 SNPs (including two families and two genus of the gut microbiome) were available for subsequent MR analysis. The IVW method indicated a causal association of *Family_Ruminococcaceae* (OR = 0.46 [95% CI, 0.26–0.82]; $p = 0.009$) and *Genus_Porphyromonadaceae* (OR = 0.59 [95% CI, 0.42–0.83]; $p = 0.003$) with HCC, whilst *Family_Porphyromonadaceae* (OR = 0.36 [95% CI, 0.14–0.94]; $p = 0.036$) and *Genus_Bacteroidetes* (OR = 0.55 [95% CI, 0.34–0.90]; $p = 0.017$) with ICC (Table 1 and Figure 3). The weighted median method showed consistent conclusions in the causal association analysis of *Family_Ruminococcaceae* (OR = 0.32 [95% CI, 0.13–0.79]; $p = 0.013$) with HCC, and *Genus_Bacteroidetes* (OR = 0.50 [95% CI, 0.26–0.96]; $p = 0.038$) with ICC (Table 1 and Figure 3). Further details of the relevant SNPs are present in Supplementary document.

The scatter plot also revealed the protective effect of *Family_Ruminococcaceae* and *Genus_Porphyromonadaceae* on HCC, *Family_Porphyromonadaceae* and *Genus_Bacteroidetes* on ICC respectively (Figure 4A–D). Leave-one-out sensitivity test showed that no single SNP had a dominant effect on the overall assessment (Figure 4 E–H).

Cochran's *Q* statistics suggested no significant heterogeneity amongst the selected SNPs (Table 1). However, when we performed a horizontal pleiotropy test, i.e., Egger intercept test, we detected potential horizontal pleiotropy (Egger intercept = −0.13, se = 0.06,

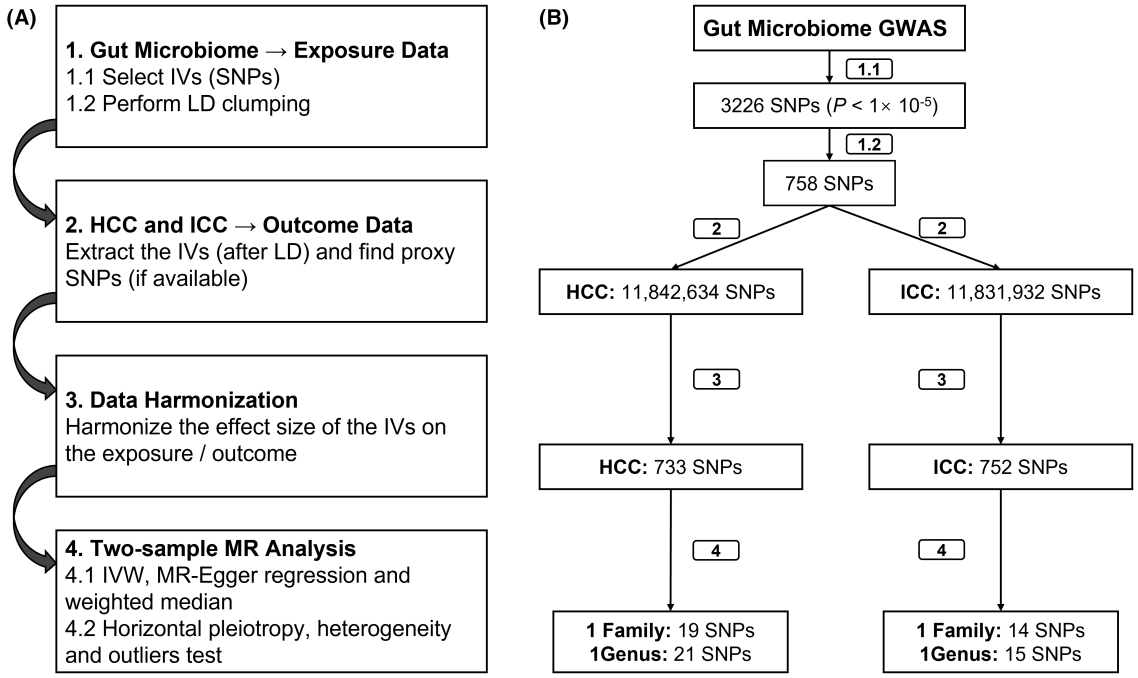

FIGURE 2 Flow chart of Mendelian randomization.

TABLE 1 The association of increased relative abundance of gut microbiome with liver cancer

Exposure	Outcome	SNP (*n*)	Methods	Beta	Se	OR (95% CI)	*p*-value	Cochran's Q			Horizontal pleiotropy			*F*-statistic (median)
								Q	Q_df	Q_pval	Egger intercept	Se	*p*-value	
F_Ruminococcaceae	HCC	19	MR-Egger	−0.69	0.61	0.50 (0.15–1.64)	.268	14.71	17	0.6164	−0.01	0.07	.8793	22.12
			IVW	−0.78	0.30	0.46 (0.26–0.82)	.009	14.73	18	0.6860	/	/	/	
			Weighted median	−1.14	0.46	0.32 (0.13–0.79)	.013	/	/	/	/	/	/	
			MR-RAPS	−0.69	0.30	0.50 (0.28–0.91)	.024	/	/	/	/	/	/	
			MR-PRESSO	−0.67	0.28	0.51 (0.30–0.88)	.025	/	/	/	/	/	/	
			Radial MR	−0.66	0.28	0.52 (0.30–0.89)	.017	/	/	/	/	/	/	
G_Porphyromonadaceae	HCC	21	MR-Egger	0.04	0.31	1.04 (0.56–1.91)	.911	17.45	19	0.5597	−0.13	0.06	.0487	20.35
			IVW	−0.52	0.17	0.59 (0.42–0.83)	.003	21.88	20	0.3469	/	/	/	
			Weighted median	−0.14	0.24	0.87 (0.55–1.38)	.558	/	/	/	/	/	/	
			MR-RAPS	−0.51	0.17	0.60 (0.43–0.84)	.003	/	/	/	/	/	/	
			MR-PRESSO	−0.47	0.16	0.62 (0.46–0.85)	.007	/	/	/	/	/	/	
			Radial MR	−0.48	0.16	0.62 (0.45–0.85)	.003	/	/	/	/	/	/	
G_Porphyromonadaceae (Without outliers)	HCC	19	MR-Egger	−0.11	0.61	0.90 (0.27–2.98)	.862	12.57	17	0.7647	−0.12	0.10	.2550	20.35
			IVW	−0.79	0.20	0.45 (0.31–0.68)	<.001	13.95	18	0.7321	/	/	/	
			Weighted median	−0.60	0.28	0.55 (0.32–0.96)	.034	/	/	/	/	/	/	
			MR-RAPS	−0.72	0.20	0.49 (0.33–0.73)	<.001	/	/	/	/	/	/	
			MR-PRESSO	−0.69	0.17	0.50 (0.36–0.70)	<.001	/	/	/	/	/	/	
			Radial MR	−0.69	0.17	0.50 (0.36–0.70)	<.001	/	/	/	/	/	/	
F_Porphyromonadaceae	ICC	14	MR-Egger	−0.01	1.48	0.99 (0.05–18.09)	.994	13.21	12	0.3542	−0.12	0.16	.4890	21.08
			IVW	−1.01	0.48	0.36 (0.14–0.94)	.036	13.77	13	0.3904	/	/	/	
			Weighted median	−0.15	0.66	0.86 (0.24–3.12)	.818	/	/	/	/	/	/	
			MR-RAPS	−0.97	0.48	0.38 (0.15–0.98)	.045	/	/	/	/	/	/	
			MR-PRESSO	−0.93	0.46	0.40 (0.16–0.98)	.065	/	/	/	/	/	/	
			Radial MR	−0.93	0.46	0.40 (0.16–0.98)	.044	/	/	/	/	/	/	

TABLE 1 (Continued)

Exposure	Outcome	SNP (*n*)	Methods	Beta	Se	OR (95% CI)	*p*-value	Cochran's Q			Horizontal pleiotropy			*F*-statistic (median)
								Q	Q_df	Q_pval	Egger intercept	Se	*p*-value	
G_Bacteroidetes	ICC	15	MR-Egger	0.15	1.09	1.16 (0.14–9.94)	.892	7.60	13	0.8688	−0.13	0.19	.4978	20.52
			IVW	−0.59	0.25	0.55 (0.34–0.90)	.017	8.08	14	0.8849	/	/	/	
			Weighted median	−0.69	0.33	0.50 (0.26–0.96)	.038	/	/	/	/	/	/	
			MR-RAPS	−0.56	0.24	0.57 (0.35–0.91)	.020	/	/	/	/	/	/	
			MR-PRESSO	−0.55	0.16	0.58 (0.35–0.91)	.003	/	/	/	/	/	/	
			Radial MR	−0.55	0.16	0.58 (0.42–0.79)	.001	/	/	/	/	/	/	

$p = 0.0487$) of SNPs in *Genus_Porphyromonadaceae*. Although MR-PRESSO failed to detect outliers in *Genus_Porphyromonadaceae*, Radial MR succeeded in detecting two outliers (rs117888257 and rs73844220). After removing the two outliers, we re-performed all analyses of *Genus_Porphyromonadaceae* with HCC, the conclusions were the same as previous and the horizontal pleiotropy disappeared (Table 1). MR-RAPS provided robust results in the complicated features of the gut microbiome, HCC and ICC (Table 1). MR-PRESSO and Radial MR detected no outliers in *Family_Ruminococcaceae*, *Family_Porphyromonadaceae* and *Genus_Bacteroidetes*, and these two methods provided reliable conclusions as a sensitivity analysis (Table 1). The above findings suggested that the increased relative abundance of the four gut microbiome classifications has a protective effect on HCC and ICC.

3.2 | Case–control study

3.2.1 | Baseline demographic information and clinical features

The case–control study collected faecal samples from 287 participants, including 184 HCC patients, 63 ICC patients and 40 healthy controls. Continuous variables did not meet the preconditions for ANOVA and *t*-test, so the Kruskal-Wallis test and Mann–Whitney test were utilized. The comparison of all participants' baseline demographic and clinicopathologic features was displayed in Table 2.

3.2.2 | α-diversity and β-diversity

α-diversity and β-diversity are essential indicators in describing the composition and distribution of microorganisms. Sequences ≥100% similarity were classified as the same feature. Venn diagrams demonstrated that each group was distinct from the other two groups, with independent gut microbiota at the phylum and genus levels (Figure 5A,B). We selected the Chao1 index, Shannon index and Simpson index as further descriptions of α-diversity. Box plot of the Chao1 index revealed that healthy controls versus HCC patients ($p = 5.1 \times 10^{-8}$), healthy controls versus ICC patients ($p = 0.025$) and HCC patients versus ICC patients ($p = 0.022$) were all statistically significant (Figure 5C). Meanwhile, the box plot of the Shannon index suggested statistical significance between healthy controls versus HCC patients ($p = 6.7 \times 10^{-5}$), and the Simpson index suggested statistical significance in the HCC patients versus the healthy control ($p = 0.0016$) and HCC versus ICC patients ($p = 0.043$) respectively (Figure 5D,E). We performed PCoA analysis based on the weighted UniFrac distance and NMDS analysis to assess β-diversity. The PCoA analysis indicated that the three groups reached the statistical significance ($R = 0.1454$, $p = 0.001$) gut microbiological composition (Figure 5F) and the NMDS analysis ($R = 0.1454$, Stress = 0.27, $p = 0.001$) obtained consistent conclusions (Figure 5G).

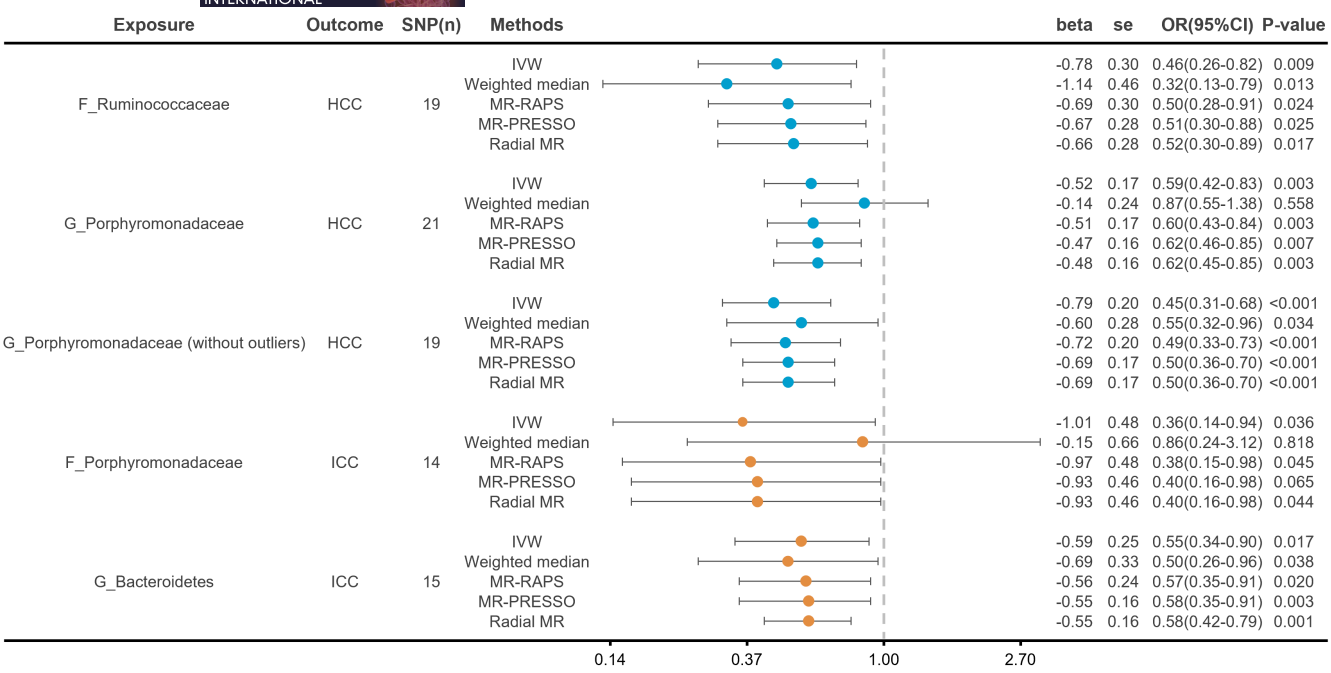

FIGURE 3 Forest plot of the association between gut microbiome & HCC and ICC.

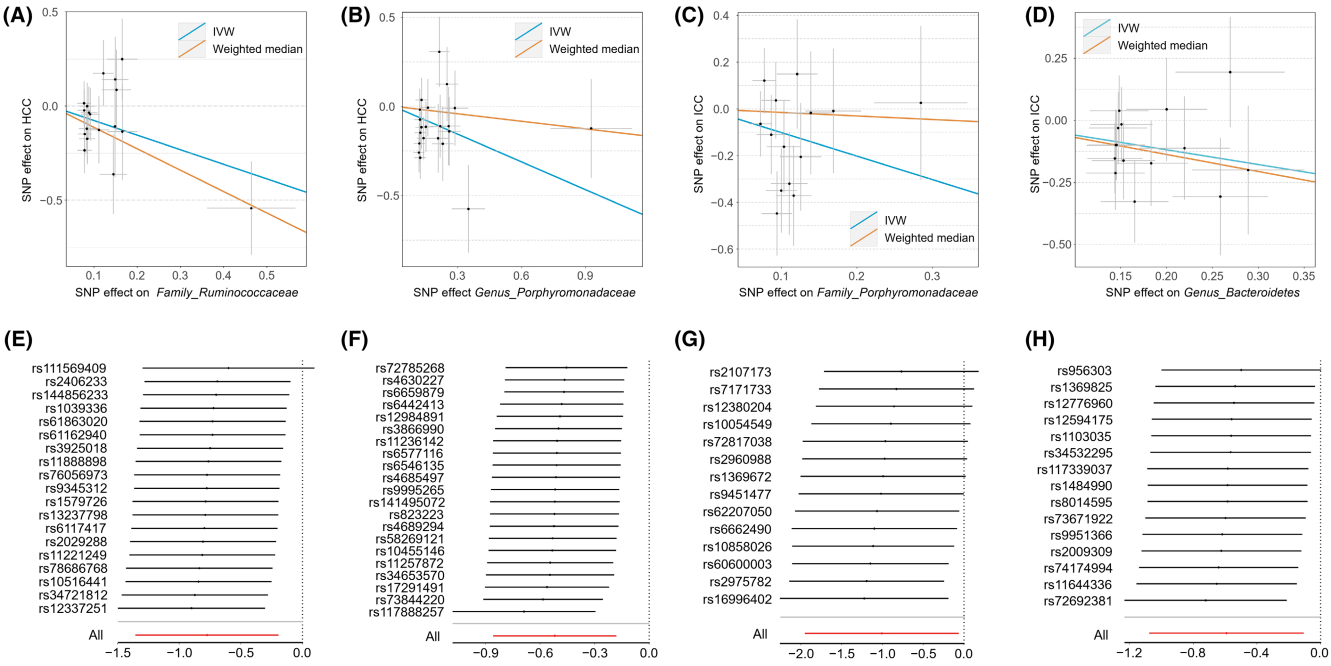

FIGURE 4 Scatter (A–D) and leave-one-out sensitivity analysis (E–H) of the association between gut microbiome & HCC and ICC.

3.2.3 | Validation of MR

Once the direction of the causal association was confirmed by MR, we could perform validation with the data obtained from the case–control study. We extracted the relative abundance of the *Family_Ruminococcaceae*, *Genus_Porphyromonadaceae*, *Family_Porphyromonadaceae* and *Genus_Bacteroidetes* according to the corresponding groups and compared them by bar plots. Healthy controls had a higher relative abundance of *Family_Ruminococcaceae* ($p = 0.00033$) than the HCC patients (Figure 6A). Simultaneously, the relative abundance of *Family_Porphyromonadaceae* ($p = 0.0055$) and *Genus_Bacteroidetes* ($p = 0.021$) were also higher in healthy controls compared to ICC patients (Figure 6B,C). By validating the findings of MR with sequencing data, the bar plots showed a higher relative abundance of the relevant gut microbes in healthy controls, suggesting that these microbiomes exhibit protective traits during

TABLE 2 Demographic and clinicopathologic features of participants in the case–control study

Characteristic	Healthy control ($n = 40$)	HCC patients ($n = 184$)	ICC patients ($n = 63$)
Age, median (IQR), y	55.0 (43.0, 63.5)	62.0 (53.0, 68.0)	70.0 (62.0, 74.0)
Gender, No. (%)			
Male	8 (20)	158 (86)	32 (51)
Female	32 (80)	26 (14)	31 (49)
BMI, median (IQR), kg / m^2	23.0 (23.0, 23.7)	23.4 (21.3, 25.2)	22.8 (20.3, 24.4)
HBV-infected history, No. (%)			
Yes	2 (5)	104 (57)	10 (16)
No	38 (95)	80 (43)	53 (84)
Cirrhosis history, No. (%)			
Yes	0 (0)	103 (56)	9 (14)
No	40 (100)	81 (44)	54 (86)
Smoking history, No. (%)			
Yes	6 (15)	80 (44)	19 (30)
No	34 (85)	104 (56)	44 (70)
Drinking history, No. (%)			
Yes	8 (20)	71 (39)	13 (21)
No	32 (80)	113 (61)	50 (79)
AFP, median (IQR), ng/ml	NA	10.2 (3.7, 133.1)	3.0 (2.3, 4.2)
CEA, median (IQR), μg/L	NA	2.2 (1.5, 3.3)	4.1 (1.9, 19.7)
CA19-9, median (IQR), U/ml	NA	16.5 (8.6, 37.2)	145.4 (37.7, 858.5)

Abbreviations: AFP, alpha-fetoprotein; BMI, body mass index; CA 19–9, carbohydrate antigen 19–9; CEA, carcinoembryonic antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; ICC, intrahepatic cholangiocarcinoma; IQR, interquartile range; NA, not available.

the development of HCC or ICC. This result was in line with the conclusions derived from MR. Since our sequencing data indicated that the relative abundance of *Genus_Porphyromonadaceae* was 0 in over 90% of healthy controls and HCC patients, we failed to conclude a difference between these two groups.

4 | DISCUSSION

In this study, we identified that the genetically increased relative abundance of four gut microbiomes is related to a reduced risk of liver cancer, including *Family_Ruminococcaceae* and *Genus_Porphyromonadaceae* for HCC, *Family_Porphyromonadaceae* and *Genus_Bacteroidetes* for ICC respectively. To our knowledge, the present work is the first report on the application of the MR concept to explore the causality between the gut microbiome and liver cancers. Although disease phenotypes in populations could be influenced by genetic inheritance and environmental factors, MR was analysed directly using genetically related GWAS data. Moreover, we performed strict quality control on the included SNPs that avoided the influence of confounding factors and reverse causality. Especially, cirrhosis, HBV infection and NAFLD were significant risk factors for primary liver cancer; we identified all IVs with PhenoScanner and PheWAS databases to avoid confounding effects. Causal associations focus strongly on the temporal sequence of cause and effect. After confirming the causality between the gut microbiome and HCC or ICC by the MR analysis, we verified the result by a case–control study. We successfully validated the difference in the relative abundance of the three gut microorganisms between healthy controls and liver cancer patients. However, we failed with *Genus_Porphyromonadaceae* because of the limitations of sequencing data. The gut microbiome represents a vast aggregation of microorganisms in the human digestive tract, capable of influencing the physiological functions of the gastrointestinal tract and the liver[10,40] and interacts with almost all human cells.[41] The gut microbiome is charged with essential physiological functions of the human organism, including bile acid metabolism,[10] synthesis of essential vitamins[40] and regulation of the immune system.[42] As a result of the unique anatomical position of the liver and gut, it can cause disease once the balance between them is disturbed. Liver cancer is generally a consequence of the progress of chronic liver diseases, implying a low incidence of HCC in the absence of any liver diseases.[21] Gut microbiome dysregulation and/or translocation have been observed in the early stages of chronic liver disease, leading to the deterioration of the disease if not controlled.[20,23] The gut microbiome with the metabolites is a low-level exposure to the liver, generally described as microbiota-associated molecular patterns (MAMPs). MAMPs show high levels when the gut microbiome is dysregulated, resulting in the induction of hepatocarcinogenesis by diethylnitrosamine (DEN) and carbon tetrachloride (CCl4) by binding lipopolysaccharides (LPS) and their receptors Toll-like receptor 4

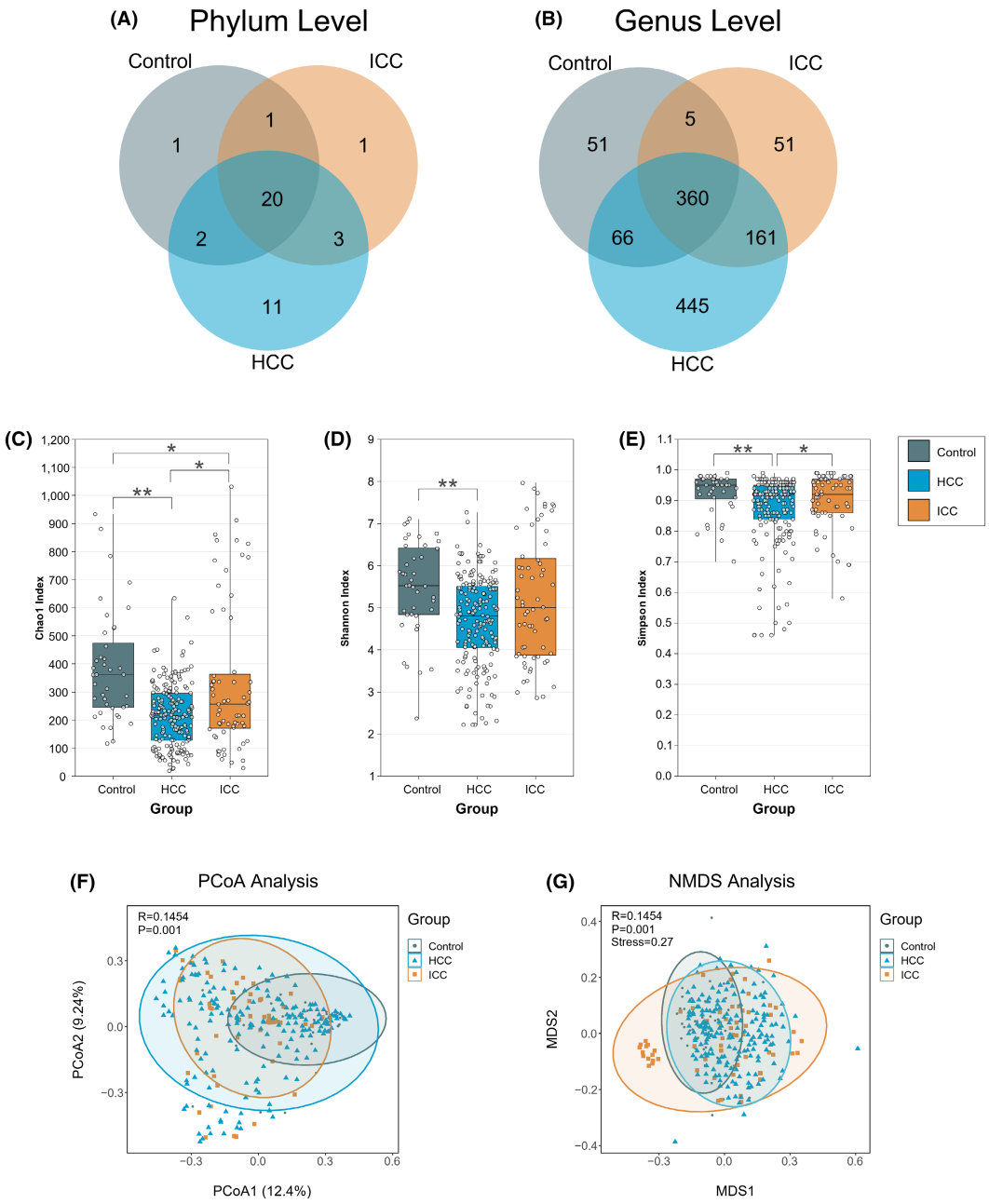

FIGURE 5 Differences in gut microbiome composition and distribution in healthy controls, HCC patients and ICC patients. Venn diagrams showed the composition of different groups of gut microbiome at the phylum level (A) and genus level (B). α-diversity was described by the Chao1 index (C), Shannon index (D) and Simpson (E). β-diversity was described by principal coordinates analysis (PCoA) based on weighted UniFrac distance metric (F) and non-metric multidimensional scaling (NMDS) analysis (G). $^{*}p < .05$, $^{**}p < .01$.

(TLR4).[23] Further, TLR4 has been proven to cause liver damage.[43,44] In addition, immune regulation is crucial in the multiple functions of the gut microbiome because it is frequently associated with malignancy.[45] The gut microbiome can directly or indirectly promote or inhibit tumorigenesis by secreting endogenous metabolites, changing the intestinal barrier and regulating the body's immune response.[46] Bile acid metabolism mediated by the gut microbiome can regulate liver cancer via natural killer cells or T cells and other pathways, such as immunoglobulins A (IgA), interleukins (IL-6, 10, 12 and 33) and transforming growth factors (TGFs).[47,48]

Focus on the gut microbiome involved in this study, MR revealed the causal association between *Ruminococcaceae*, *Porphyromonadaceae*, *Bacteroidetes* and primary liver cancer respectively. The 16s rRNA sequencing data was crucial for validating the MR findings. However, the complex mechanism between the gut microbiome and primary liver cancer needs further exploration. The aetiology and progression of liver diseases can be partially predicted by the metabolites or metabolomes of the gut microbiome.[49] *Ruminococcaceae* is one of the most dominant taxa in the *Phylum_Firmicutes*.[50] It reported that the relative abundance

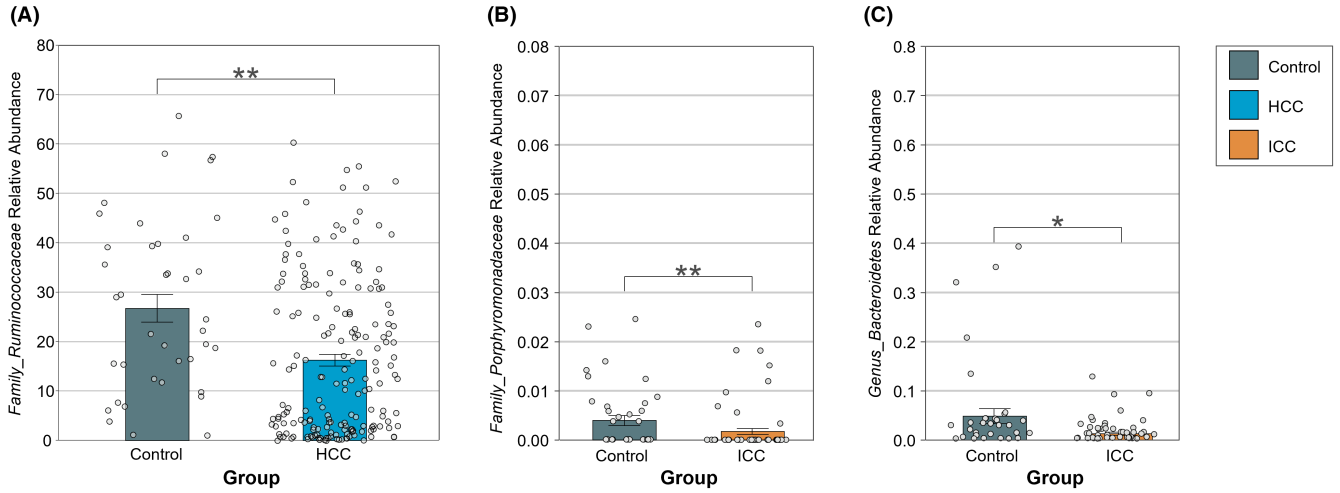

FIGURE 6 Bar plots to validate Mendelian randomization. Comparative relative abundance of Family_Ruminococcaceae (A) in healthy controls and HCC patients; comparative relative abundance of Family_Porphyromonadaceae (B) and Genus_Bacteroidetes (C) in healthy controls and ICC patients. $^{*}p < .05$, $^{**}p < .01$.

of *Ruminococcaceae* decreased in mice models of a high-fat diet and patients with non-alcoholic steatohepatitis (NASH).[50] In patients with colorectal cancer, *Ruminococcaceae* is depleted, suggesting that *Ruminococcaceae* may be a protective flora.[51] A study of gut microbiome and metabolites with significant fibrosis in non-obese NAFLD patients demonstrated that bile salt hydrolase (BSH) was downregulated in abundance in subjects, whilst the *Ruminococcaceae* was the major contributor to BSH.[52] Amongst the four gut microbiomes we explored in this study, the *Genus_Porphyromonadaceae* is categorized as the *Family_Porphyromonadaceae* (https://lpsn.dsmz.de/). HCC and ICC are highly heterogeneous primary liver cancers; MR analysis identified that *Porphyromonadaceae* is associated with HCC and ICC. In an Italian cohort study, researchers assessed visceral fat content and metabolic status in different elderly groups; the results showed that visceral fat content was lower, and the metabolic status was healthier in the elderly group with a higher abundance of *Porphyromonadaceae*.[53] The study showed that *Porphyromonadaceae* positively correlated with aromatic amino acid metabolism, ammonia metabolism and oxidative stress indicators to affect cirrhosis's progression.[54] Further rigorous analysis is necessary to exclude confounding factors for identification. A study on gut microbial characterization of Chinese patients with cirrhosis reported a significant reduction in the proportion of *Bacteroidetes*.[55] Meanwhile, *Bacteroidetes* can produce a metabolite called sphingolipid to regulate gut ecology, and in the germ-free mouse model lacking *Bacteroidetes* colonization, mice are more prone to develop gut inflammation.[56] *Bacteroidetes* also contributed to energy homeostasis by expressing glycosyltransferases, glycoside hydrolases and polysaccharide lyases, associated with the development of NASH in animal models.[57]

The strength of this study is to explore the causal association between the gut microbiome and liver cancer by the MR method, which can avoid confounding factors and reverse causality. Our conclusions were corroborated by multiple sensitivity analyses, suggesting higher robustness of the findings. In addition, by using the powerful Radial MR algorithm to detect outliers efficiently, we can improve the accuracy of the two-sample MR, which fits a radial IVW model using modified second-order weights.[58] However, our study is still subject to some limitations. First, the SNPs we selected as IVs may still be influenced by potential horizontal pleiotropy. Genetic inheritance, lifestyle, and environmental factors can change the gut microbiome, resulting in minor variance explained by IVs. The current study cannot judge whether IVs are relevant to confounding. Second, we are unable to explore the whole gut microbiome from phylum to genus and, therefore, we may have missed other gut microbes that were causally associated with HCC and ICC, especially the risky species. Third, the participants in the GWAS summary statistics selected in this study are from the European populations; extrapolation of the study conclusions to other ethnic populations may be limited, even though we have partially validated participants from Asian populations.

Our findings based on MR supported a potential causality between the gut microbiome and liver cancer and were partially validated with sequencing data. These gut microbes could be potential for HCC and ICC prevention and treatment. At the same time, the bidirectional causal relationship between gut microbes and liver cancer requires higher quality GWAS data, and further investigations into the underlying mechanisms are necessary.

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CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose.

DATA AVAILABILITY STATEMENT

All data and materials supporting the findings of this work are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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