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ORIGINAL ARTICLE

Intratumoral Microbe Correlated with Expression of DNA Methylation Genes in Hepatocellular Carcinoma

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Abstract

Background Intratumor microbiota are increasingly implicated in cancer pathogenesis, with emerging evidence suggesting their role in regulating DNA methylation pathways in hepatocellular carcinoma (HCC). This study investigates the interactions between specific bacterial genera and key DNA methylation-related genes in HCC tissues.

Methods Tumor and adjacent normal tissues from 72 HCC surgical patients were analyzed. Bacterial composition was characterized via 16SrRNA sequencing, while quantitative analysis assessed the abundance of differential bacterial genera and expression of DNA methylation pathway genes linked to HCC outcomes.

Results Six bacterial genera exhibited differential abundance between tumor and normal tissues: *Propionibacterium*, *Mycoplasma*, *Variovorax*, *OPB41*, *unclassified_Bifidobacteriaceae*, and *Rikenellaceae*. Key gene–microbiota correlations included: Negative associations of *Propionibacterium* with SPOCD1 and EOMES; Negative associations of Mycoplasma with BMI1 and EZH2; Negative correlations of *Pseudomonas* with BMI1, EOMES, EZH2, and SPOCD1. Additionally, *Pseudomonas* abundance was negatively correlated with both *Propionibacterium* and *Mycoplasma*.

Conclusion The identified microbiota–gene interactions reveal potential mechanistic links between intratumor flora and HCC progression via DNA methylation regulation. These findings highlight novel targets for further exploration of HCC therapeutic strategies.

Keywords Hepatocellular carcinoma · Intratumoral microbe · Methylation · DNA · Flora

Introduction

Hepatocellular carcinoma (HCC) originating from abnormal liver cells exhibits a propensity for constant cellular changes that initially manifest as benign liver tumors [1]. However, over time, these benign tumors may progress into malignant HCC [2]. With a low likelihood of cure, HCC is a relatively lethal condition, particularly when diagnosed at an advanced stage [3]. According to the World Health Organization, HCC ranks third in terms of fatality among cancers, causing approximately 800,000 deaths annually [4]. The highest incidence of HCC is observed in Asia, Africa, and the Pacific region, with countries such as China, Japan, and Vietnam experiencing relatively high rates. HCC possess multiple risk factors, including hepatitis B and C virus infections, chronic alcohol consumption, cirrhosis, hypertension, obesity, and diabetes.

Microorganisms, notably viruses, strongly correlate with the development of HCC [5]. Hepatitis B virus (HBV) infection ranks among the most prominent causes [6]. Prolonged HBV infection can induce chronic inflammation of hepatocytes and liver fibrosis, ultimately culminating in HCC. Similarly, hepatitis C virus (HCV) infection elicits chronic liver cell inflammation and fibrosis, thereby elevating the risk of HCC [7, 8]. Noteworthy connections exist between specific types of human papillomavirus (HPV)

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infection and HCC, particularly in immunosuppressed patients and those who underwent liver transplantation [9, 10].

In recent years, in addition to viral agents, studies have revealed associations between gut microorganisms and HCC, leading to the concept of the gut microbiome [11–15]. Dysregulation of the gut microbiome potentially contributes to the development and progression of hepatitis, a major precursor to HCC [16, 17]. Certain beneficial microbiome species aid in regulating the intestinal immune system, inhibiting the growth of pathogenic bacteria, and reducing intestinal toxins. Conversely, an imbalanced microbiome may trigger an elevated inflammatory response, thereby promoting hepatitis development [18]. Liver fibrosis and cirrhosis represent premalignant lesions, and it is plausible that abnormalities in gut microbiome contribute to their progression [19]. Evidence indicates that dysregulation of specific microbiome species is linked to exacerbated liver fibrosis and cirrhosis [20]. This dysregulation includes over proliferation of harmful microbiome, reduction of beneficial microbiome, and disruption of their metabolic byproducts, potentially intensifying inflammatory responses and fibrosis [21, 22]. Moreover, these microorganisms may impact HCC development through the production of harmful substances and modulation of immune responses.

Furthermore, emerging evidence suggests the presence of intratumoral bacteria that may be implicated in HCC carcinogenesis [23]. Remarkably, nearly all types of human cancers exhibit a distinct intratumoral bacterial composition. There have been notable findings highlighting the crosstalk between the tumor microbiota and the gut microbiota, which can potentially modulate the host's immune response. One recent study observed the presence of multiple taxonomic signatures of bacterial DNA in liver tissues of individuals with nonalcoholic fatty liver disease (NAFLD). In another investigation involving 156 HCC tissues, it was consistently observed that the *Aspergillus*, *Bacteroidetes*, *Bacillus* and *Actinobacteria* were presented in para-tumoral and HCC tissues [24]. Additionally, *Gammaproteobacteria*, *Streptococcaceae* and *Lactococcus* exhibited a particular enrichment in HCC, were identified as marker for HCC and cirrhosis, while the *Staphylococcus* was associated with the pathologic features of HBV infection. In another study of 28 cases of Primary Liver Cancer, bacteria of the Pseudomonas family, were found to have decreased abundance in tumor tissue and were linearly associated with prognosis [25]. In contrast, a separate study involving 47 cases identified *Oscillospira*, *Mucispirillum*, *Helicobacter, Roseburia, Ruminococcus*, and *Anaerotruncus* as the differential organisms between normal and HCC tissues [26]. It was also found that specific some bacterial abundance was associated with the expression of DNA methylation genes which play critical role in HCC [26, 27].

While previous studies have identified the primary bacteria present in HCCtumors and their correlation with epigenetic gene expression, none of them have explored the relationship between these bacteria and HBV infection, an important factor in the development of HCC. Therefore, this study was to investigate bacterial DNA in cancerous and paracancerous tissues of 72 hepatocellular carcinoma cases using 16 s ribosomal RNA sequencing. By analyzing the differences between cancerous and paracancerous tissues, we aimed to determine the association between bacterial abundance and HBV infection. Additionally, the expression of HBV-p22, HBx and the gene expression of the Wnt signaling pathway and its associated epigenetic regulatory pathway were examined to further assess this relationship.

Material and Methods

Patients

We assembled a cohort of HCC patients (72) from The First Affiliated Hospital of Fujian Medical University Hospital in Fujian Province, China from 2015 to 2016. Each patient provided representative tumor tissues and adjacent non-tumor tissues for analysis.

Sample Collection

For each sample, we collected sufficient tissue specimens for 16S rRNA sequencing and quantitative gene analysis. We used the QIAamp DNA kit from QIAGEN (California, USA) to extract total microbial DNA. Initially, the tissue cells were lysed to release the DNA. Subsequently, we purified the DNA from other impurities using the buffer and purification steps provided in the kit. Following extraction, the DNA underwent a washing and drying process to ensure sample purity and quality. DNA samples are applied for NanoDrop ND-1000 instrument to check the quality. This instrument measures the spectral absorption of the samples to determine DNA concentration and purity. Finally, we immediately froze and stored the extracted DNA samples at -20 °C to maintain stability and preserve integrity for subsequent experimental analysis.

PCR Amplification, Sequencing, and Data Processing

We conducted PCR amplification and sequencing using the following steps. Initially, we used a forward primer (5-GTGCCAGCMGCCGCGGTAA-3) and a reverse primer (5-GGACTACHVGGGTWTCTAAT-3) to amplify the V4 region of the bacterial 16S rRNA gene in diluted DNA samples. After completing the PCR reaction, we purified and quantified the product, and equimolarly mixed

the samples. Next, we sequenced the samples with the illumina MiSeq platform provided by China Baxter Biotechnology Co., using a 2×250 base pair kit. Prior to sequencing, we processed the PCR products. To differentiate the multiplexed reads, we added unique molecular barcodes to the PCR reactions and merged them using the cross-reference library generation process. We accomplished this step using USEARCH v7.0.1090 software. The sequence datasets were released in NCBI PRJNA1006188.

Bioinformatics Analysis

To perform the bioinformatics analysis, we followed these steps. Initially, we randomly selected an equal number of reads in the samples and filtered the operational taxonomic units (OTUs) using the UPARSE pipeline. We identified and removed chimeric sequences. Next, we classified the 16S rRNA gene sequences using the RDP classifier and mapped the community abundance of each sample using the 16S rRNA ribosomal RNA manifestation database to determine the community abundance and sequencing data. To assess bacterial diversity, we conducted sample-based OTU analysis and expressed it using computational metrics implemented in the R package vegan. These metrics provide insights into the bacterial diversity profile in the samples. Principal Coordinate Analysis (PCA) is performed to understand the dissimilarities and similarities between samples. Finally, we used Linear Discriminant Analysis (LDA) effect sizes to determine statistically significant differences. This helped us identify taxa of statistical significance. Through these bioinformatics analysis methods, we gained a comprehensive understanding of the community composition and bacterial diversity of our samples and conducted statistical comparisons and analyses.

RNA Extraction and qRT-PCR

Total RNA was extracted from 72 paired HCC tumor and adjacent normal tissues using TRIzol reagent (Invitrogen, USA), following the manufacturer's protocol. RNA purity and concentration were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). cDNA was synthesized from 1 μg of RNA using the PrimeScript RT Reagent Kit (Takara Bio, Japan). qRT-PCR was performed in triplicate with SYBR Green Master Mix (Applied Biosystems) on a QuantStudio 5 Real-Time PCR System. The primer sequences for BMI1, EOMES, EZH2, SPOCD1, and GAPDH (internal control) are listed in Supplementary Table S1. Gene expression was normalized to GAPDH using the $2 - \Delta\Delta Cq$ method. Primer list was shown in Table S1.

Gene Ontology (GO) Analysis for DNA Methylation Gene Identification

To identify the genes directly associated with the DNA methylation pathway, we performed a Gene Ontology (GO) enrichment analysis using the DAVID Bioinformatics Database (version 6.8). The analysis focused on the biological process (BP) and molecular function (MF) categories. The criteria for gene selection included:

GO Terms: Genes annotated with "DNA methylation," "chromatin modification," "methyltransferase activity," or "epigenetic regulation of gene expression."

Statistical Thresholds P-value: Genes with a GO term enrichment p-value < 0.05 (Benjamini–Hochberg corrected).

Enrichment Score: Terms with a minimum enrichment score of 1.3 (equivalent to a non-log scale fold enrichment > 2).

From the TCGA-HCC dataset, 28 genes met these criteria. These genes were further validated against the MSigDB (Molecular Signatures Database) to confirm their roles in DNA methylation pathways.

Statistical Analysis

We performed statistical analysis using SPSS 21.0 for Windows software. Differences between groups were assessed as follows:

One-way ANOVA was used to evaluate differences among three subgroups stratified by HBV infection status (HBV-positive tumor tissues, HBV-negative tumor tissues, and adjacent non-tumor tissues). This allowed us to determine whether bacterial abundance or gene expression varied significantly across HBV-related subgroups.

Wilcoxon rank sum test (for unpaired comparisons) and Wilcoxon signed-rank test (for paired comparisons) were applied to assess the differences in continuous variables between tumor tissues (HCC) and adjacent normal tissues (two groups).

Fisher's exact test was used to compare categorical variables (e.g., bacterial presence/absence, clinical parameters) between the same two groups (tumor vs. adjacent tissues).

Results

Abundance of Certain Microbes Altered in HCC

To investigate the intratumoral microbiome of hepatocellular carcinoma (HCC), we conducted a comparative analysis of microbial diversity between tumor and matched adjacent non-tumor tissue samples. Surprisingly, results did not

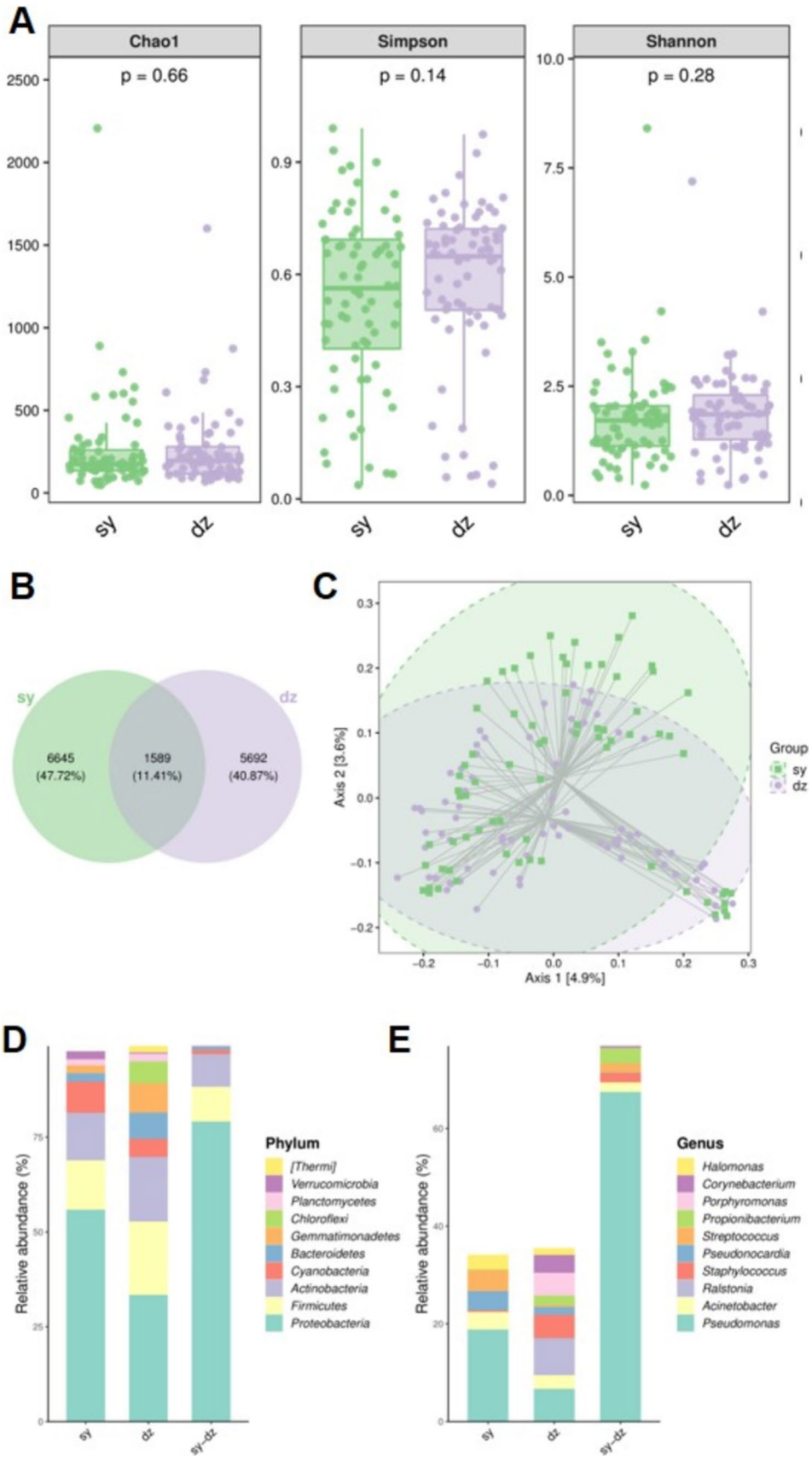


Fig. 1 General characterization of the HCC intratumoral microbiome. **A** Diversity index of intratumoral and normal tissue microbiota. Chao1, Simpson and Shannon based on different algorithms. **B** Venn diagram of OTUs in intratumoral and normal tissue microbiota. **C** Principal coordinate analysis (PCoA) of beta-diversity. Jaccard based PCoA, with ellipse. **D** and **E** Abundance of corresponding OTUs in intratumoral and normal tissue microbiota at the phylum (**D**) and genus (**E**) level. intratumoral, dz group; normal tissue, sy group

reveal any statistically significant differences in microbial diversity between these two groups, as indicated by the Chao index, Simpson index, and Shannon index (Fig. 1A). However, upon further examination using a Venn diagram, we observed that 1589 OTUs were shared between the tumor and non-tumor tissues, while 5692 OTUs were unique to HCC (Fig. 1B).

Furthermore, by assessing the beta-diversity using Principal Component Analysis (PCA), we found that the microbial communities in tumor tissues exhibited a symmetrical distribution across all samples, with some degree of overlap (Fig. 1C). The dominant bacterial phyla identified included *Proteobacteria, Firmicutes, Actinobacteria, Cyanobacteria*, and *Bacteroidetes*, collectively accounting for more than 75% of the sequences (Fig. 1D). Additionally, at the genus level, we observed that *Pseudomonas* and *Streptococcus* exhibited significant reductions in tumor tissues, possibly attributed to the antitumor effects of Pseudomonas (Fig. 1E). Similarly, Acinetobacter also showed a moderate reduction in tumor tissues. Conversely, the genera *Ralstonia, Staphylococcus, Corynebacterium*, and *Porphyromonas* were found to be significantly increased in tumor tissues (Fig. 1E).

Differentiate Bacteria Genus Between Para- and Cancer Tissue

In order to distinguish carcinoma from paracancer, we conducted an analysis called LEfSe, using a log LDA score cutoff of 2.0, to statistically identify and differentiate the important taxa of organisms (Fig. 2A). The LEfSe analysis results confirmed that the genus composition of paracancerous tissues in HCC patients were enriched with *Shigella, Synechococcus, Georgenia, Arenimonas, Selenomonas, and KD1_23*, while the cancerous tissues had higher abundance of *Propionibacterium, Ralstonia, Leptolyngbya, Marivita, and Cupriavidus*. To further validate the distinction between paired paraneoplastic and carcinomatous flora, we performed a paired t-test to analyze the differences in the horizontal abundance of genera (Fig. 2B). The results revealed that the abundance of genera such as *Propionibacterium, Mycoplasma, Variovorax, unidentified_OPB41, unclassified_Bifidobacteriaceae*, and *unclassified_Rikenellaceae* have significant differences.

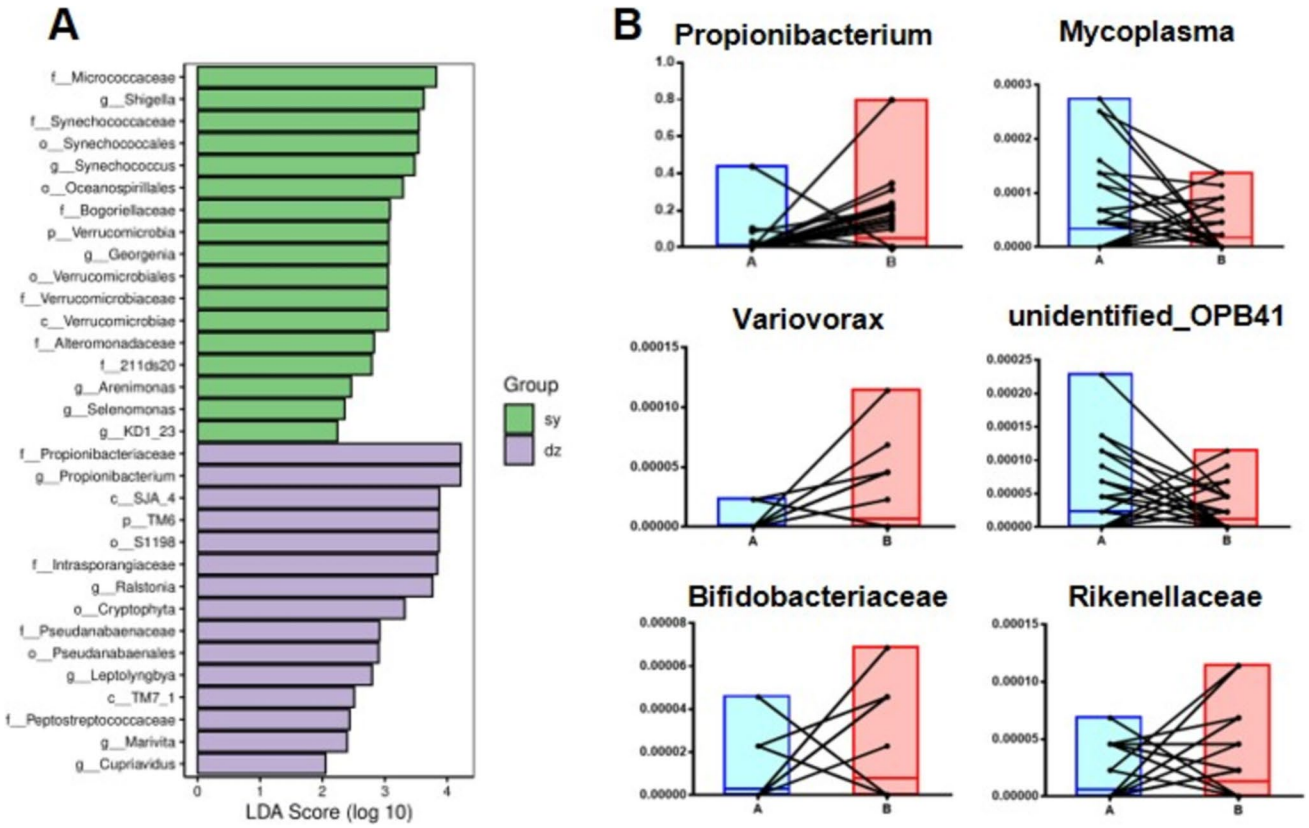
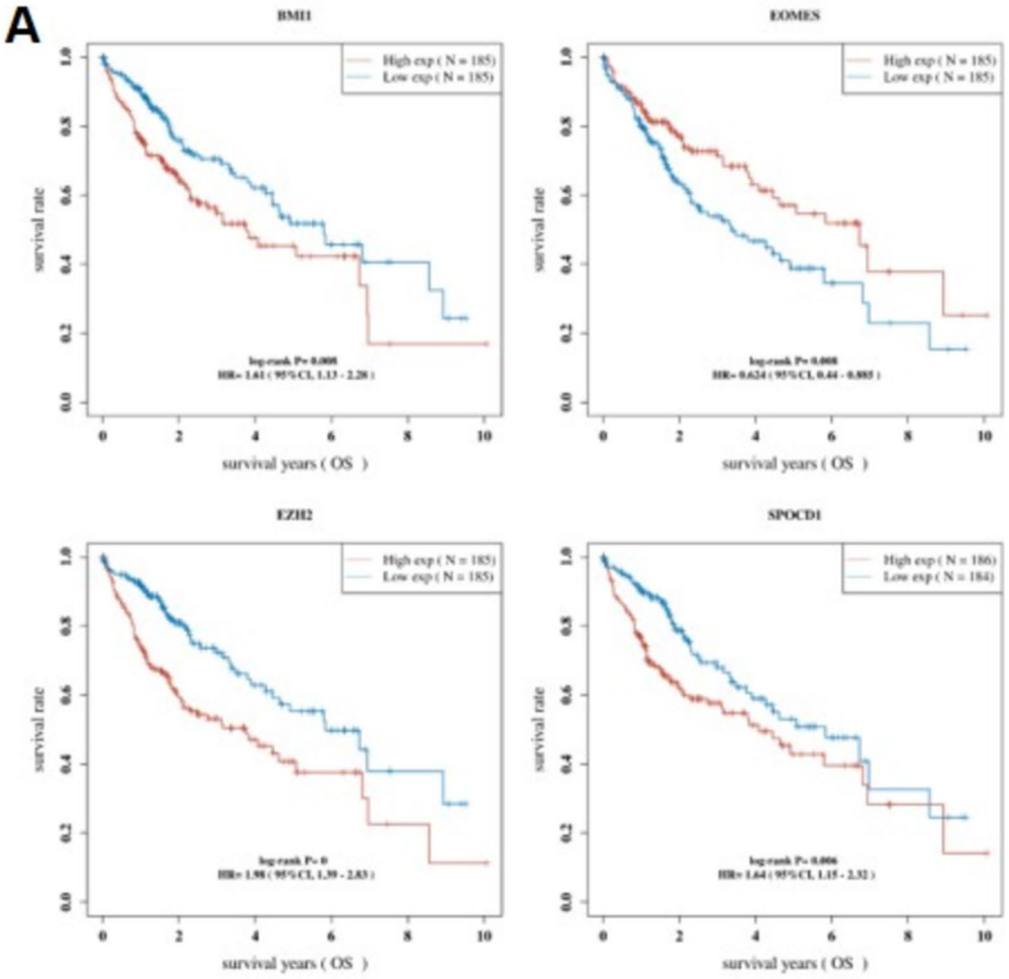
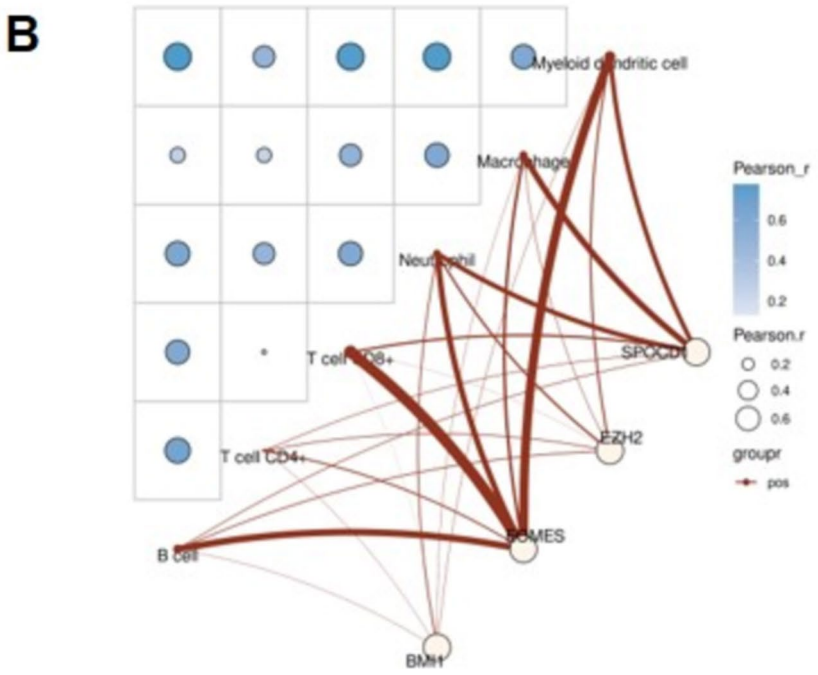


Fig. 2 Microbiome variance analysis and marker species. **A** LDA effect size (LEfSe) demonstrate the specific distribution of marker species in different subgroups of samples. **B** Differences in pairwise comparisons between intratumoral and normal tissue microbiota (t-test). intratumoral, B dz group; normal tissue, A sy group


A
BMI1
High exp (N = 185)
Low exp (N = 185)
survival rate
survival years (OS)
EOMES
High exp (N = 185)
Low exp (N = 185)
survival rate
survival years (OS)
EZH2
High exp (N = 185)
Low exp (N = 185)
survival rate
survival years (OS)
SPOCD1
High exp (N = 186)
Low exp (N = 184)
survival rate
survival years (OS)


B
Myeloid dendritic cell
Macrophage
Neutrophil
T cell CD8+
T cell CD4+
B cell
SPOCD1
EZH2
EOMES
BMI1
Pearson_r
0.6
0.4
0.2
Pearson.r
0.2
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groupr
pos

◂**Fig. 3** DNA methylation genes in HCC. **A** Kaplan–Meier curves for the OS of HCC patients based on TCGA. From 28 DNA methylation genes, 4 genes expression are significantly related to HCC OS. **B** Correlation between DNA methylation genes and immunity. intratumoral, dz group; normal tissue, sy group

Intratumor Bacteria Correlated with DNA Methylation Genes

Published literature have demonstrated a correlation between the abundance of intratumor microbiome in HCC tumors and gene expression of the DNA methylation pathway in HCC tumors [28]. To further examine the association between gene expression of the DNA methylation pathway, as identified in our data, and the characteristic flora within HCC tumors, we collected 28 genes directly associated with the DNA methylation pathway according to GO ontology annotations. Subsequently, the expression of these genes was analyzed in the TCGA database in relation to OS survival in HCC. Out of these, a total of 14 genes showed statistically significant correlations with OS survival, including *BAZ2A, BEND3, BMI1, DNMT3A, DNMT3B, EHMT2, EOMES, EZH2, HELLS, MGMT, MTA2, PPM1D, SPOCD1*, and *METTL7A*. Among them, *BMI1* ($P = 0.008$, $HR = 1.61$), *EOMES* ($P = 0.008$, $HR = 0.62$), *EZH2* ($P = 0.0002$ $HR = 1.98$), and *SPOCD1* ($P = 0.007$, $HR = 1.61$) exhibited the most significant statistical correlations. *BMI1*, *EZH2*, and *SPOCD1* were identified as beneficial factors for OS survival in HCC, while *EOMES* was identified as a risk factor (Fig. 3A). Furthermore, we investigated the relationship between the expression of *BMI1, EOMES, EZH2*, and *SPOCD1* genes and tumor immune infiltration (TIL) in HCC. We found that the expression of all four genes showed a significant positive correlation with TIL, with *EOMES* exhibiting the strongest correlation with CD8 + T cell (Fig. 3B). The genes analyzed in Fig. 4B (SPOCD1, EOMES, BMI1, and EZH2) play critical roles in hepatocellular carcinoma (HCC) pathogenesis through epigenetic regulation, immune evasion, and stemness maintenance. As a result, we decided to conduct a thorough analysis of the expression of these four genes in order to determine their correlation with the abundance of mycobacteria within HCC tumors.

To quantify the total DNA amount, we employed universal 16S rRNA primers. Additionally, we employed specific 16S rRNA primers to determine the relative abundance of three specific types of intratumoral flora: Propionibacterium, Mycoplasma, and Pseudomonas. Furthermore, we quantified the relative gene expression of *BMI1, EOMES, EZH2*, and *SPOCD1* genes in these HCC samples using real-time PCR.

By calculating Pearson correlation coefficients, we identified 12 pairs of statistically significant correlations ($P < 0.05$) (Fig. 4A). Regarding the relationships between bacteria and genes, we observed a negative correlation between Propionibacterium and *SPOCD1* as well as *EOMES*. Mycoplasma, on the other hand, showed a negative correlation with *BMI1* and *EZH2* (Fig. 4B). Conversely, Pseudomonas exhibited positive correlations with *BMI1, EOMES, EZH2*, and *SPOCD1*(Fig. 4B). Within the bacterial community, we found that Pseudomonas had a significant negative correlation with both Propionibacterium and Mycoplasma abundance.

Among these correlations, the strongest positive correlation was observed between *Pseudomonas* and *BMI1* ($r = 0.54$, $P = 0.048$), while the most pronounced negative correlation was found between *Pseudomonas* and both *Propionibacterium* ($r = -0.41$, $P = 0.0008$) and *Mycoplasma* abundance.

Discussion

The emerging research on the presence and composition of intratumoral bacteria in HCChas opened up new avenues of understanding the role of these microbes in HCC development [23]. Studies have consistently shown that HCC, along with other types of human cancers, harbors a distinct bacterial composition within the tumor itself, emphasizing the potential importance of the tumor microbiota. These findings also shed light on the intricate interplay between the tumor and gut microbiota, which can affect the host's immune response.

One particularly intriguing finding is the identification of bacterial DNA signatures in liver tissues of individuals with nonalcoholic fatty liver disease (NAFLD). This suggests that the presence of intratumoral bacteria may not be limited to HCC but could also be involved in earlier stages of liver disease. Furthermore, studies involving large cohorts of HCC tissues have revealed enriched bacterial taxa such as *Aspergillus, Bacteroidetes, Bacillus, Actinobacteria*, and *Gammaproteobacteria* in both para-tumoral and HCC tissues [26]. The presence of specific bacterial families like *Streptococcaceae* and *Lactococcus* has been associated with HCC cirrhosis, while the Staphylococcus branch has shown an association with HBV infection [24]. These findings highlight the potential role of these microbial communities in HCC development and the modulation of its pathologic features. On the other hand, different studies have identified distinct sets of bacterial taxa in HCC compared to normal tissues [25]. For instance, *Oscillospira, Mucispirillum, Helicobacter, Roseburia, Ruminococcus*, and *Anaerotruncus* have been found to be differentially abundant in HCC tissues. Additionally, the abundance of these bacteria seems to affect the expression of epigenetically regulated genes that play critical roles in HCC. This suggests a potential interaction between these bacteria and the host's genetic and

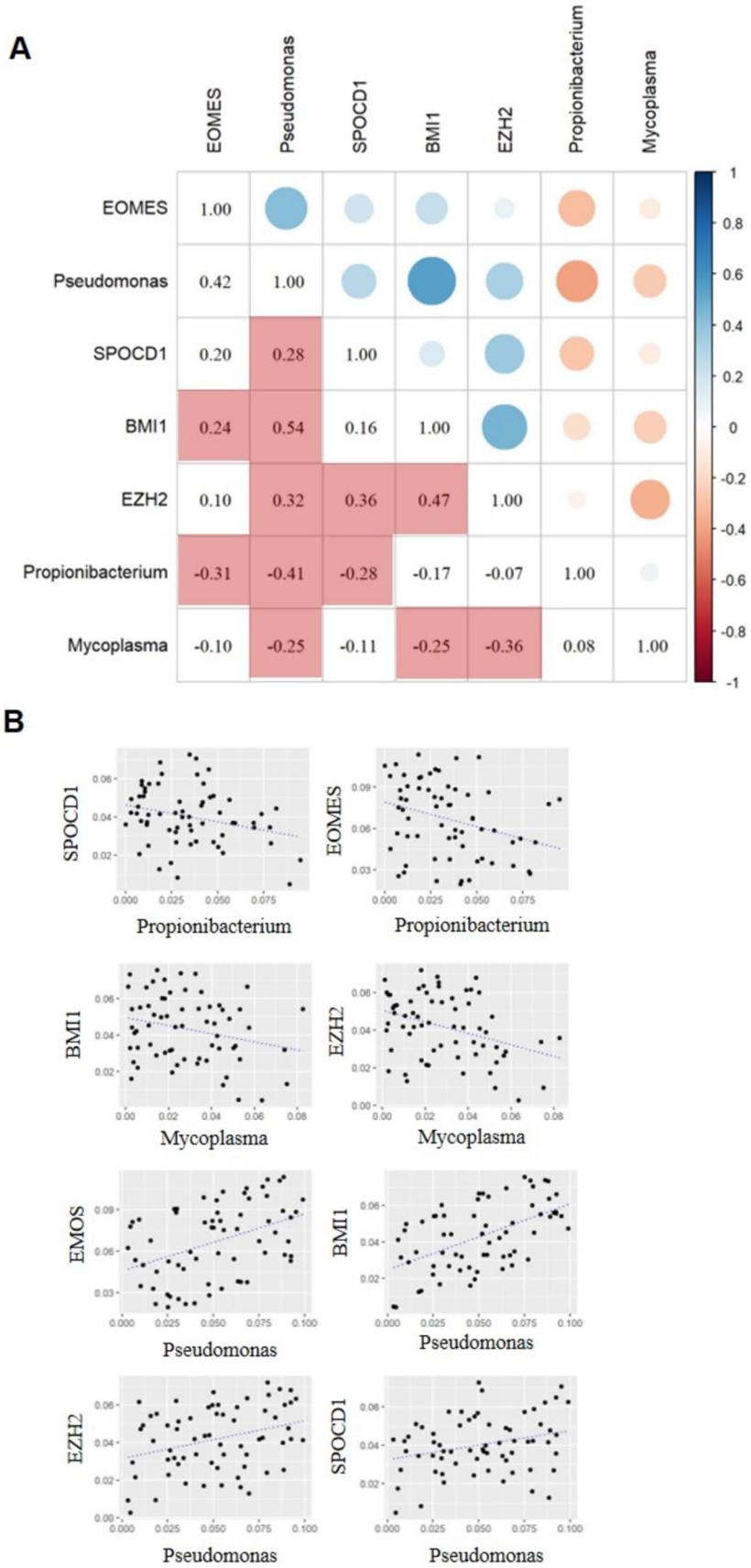

Fig. 4 Correlation between abundancy of HCC bacteria and expression of DNA methylation genes. **A** Heatmap of Pearson correlation among Propionibacterium, Mycoplasma, Pseudomonas, and DNA methylation genes includingBMI1, EZH2, SPOCD1, EOMES. The legend scale bar represents the strength and direction of correlations between bacterial abundance (Propionibacterium, Mycoplasma, Pseudomonas) and DNA methylation gene expression (BMI1, EOMES, EZH2, and SPOCD1). **B** Scatter plots indicate the Pearson correlation

epigenetic machinery, potentially influencing HCC development and progression. The identification of specific bacterial taxa associated with HCC and their correlations with pathological features and gene expression profiles provides valuable insights into the potential use of these bacteria as biomarkers for HCC diagnosis, prognosis, and therapeutic targets. However, further research is necessary to elucidate the underlying mechanisms through which intratumoral bacteria influence hepatocellular carcinoma. A deeper understanding of the complex relationship between intratumoral bacteria and HCC could potentially pave the way for novel strategies in the prevention, diagnosis, and treatment of this devastating disease. In our study, results showed a reduction in the abundance of Pseudomonas within the tumors, which is consistent with findings from previous research. However, the specific bacterial genera that were associated with HCC tumors in our samples differed somewhat from what has been reported in the literature. The differences we found, including *Propionibacterium, Mycoplasma, Variovorax, unidentified_OPB41, unclassified_Bifidobacteriaceae*, and u*nclassified_Rikenellaceae*, were not widely represented in previous studies. Among them, the abundance of Propionibacterium, Mycoplasma in the gut microbiome and the development of some tumors have been reported. It is worth noting that our sample size was relatively large compared to previous literature, and the statistical analysis yielded highly reliable and significant results. The variability in findings regarding different bacterial genera among studies suggests that intratumoral bacterial composition exhibits considerable heterogeneity. Overall, while there seems to be consistent abundance of certain bacterial species within HCC tumors, our findings indicate a greater variability and heterogeneity in the intratumoral flora.

In HCC, aberrant DNA methylation patterns are frequently observed. DNA hypermethylation, which leads to increased methylation levels, is often associated with the silencing of tumor suppressor genes. Conversely, DNA hypomethylation, characterized by decreased methylation levels, can result in the activation of oncogenes or genomic instability. Studies have identified specific genes that are commonly subjected to DNA methylation changes in HCC. In instance, the promoter regions of p*16INK4A, RASSF1A*, and *DAPK1* are frequently hypermethylated in HCC, leading to their decreased expression [29]. On the other hand, hypomethylation of oncogene *insulin-like growth factor 2* (*IGF2*) can contribute to their deregulated expression and promote HCC progression [30, 31]. Furthermore, DNA methylation patterns can serve as potential diagnostic and prognostic biomarkers in HCC. Some studies have demonstrated the utility of DNA methylation markers in the early detection of HCC or the prediction of patient outcomes [29]. These markers can be detected in blood or tissue samples, offering non-invasive approaches for HCC management. Additionally, targeting DNA methylation alterations in HCC has emerged as a potential therapeutic strategy [29]. Epigenetic drugs, such as *DNA methyltransferase* inhibitors (*DNMT* inhibitors), have shown promise in preclinical and clinical studies for the treatment of HCC [28]. In summary, DNA methylation alterations play a critical role in the development and progression of HCC. Understanding the specific genes and pathways affected by aberrant DNA methylation can provide valuable insights into the underlying mechanisms of HCC and offer potential targets for diagnosis, prognosis, and therapy [26]. Our analysis demonstrated a significant correlation between the relative abundance of Propionibacterium, Mycoplasma, and Pseudomonas with the expression of key DNA methylation factors, namely *BMI1, EOMES, EZH2*, and *SPOCD1*. Additionally, we found that these correlations were associated with the overall survival (OS) risk in patients with hepatocellular carcinoma (HCC). Specifically, Propionibacterium and Mycoplasma were found to potentially increase OS survival in HCC by downregulating the expression of *SPOCD1, EOMES, BMI1*, and *EZH2*. Conversely, Pseudomonas appeared to decrease OS survival in HCC by upregulating the expression of *SPOCD1, EOMES, BMI1*, and *EZH2*. These findings highlight the clinical significance of the association between Propionibacterium, Mycoplasma, Pseudomonas, and DNA methylation genes (*EZH2* is a histone methyltransferase) involved in HCC survival. Furthermore, they provide insights into potential mechanisms underlying these associations.

The abundance of intratumoral bacteria (e.g., Propionibacterium, Mycoplasma) may influence DNA methylation genes via bacterial metabolites (e.g., short-chain fatty acids, lipopolysaccharides) that modulate host epigenetic enzymes like DNMTs or HDACs, altering promoter methylation or histone modifications. BMI1 and EZH2 (Polycomb Repressive Complex members) are overexpressed in HCC, silencing tumor suppressors via H3K27me3. SPOCD1 promotes DNA methylation, while EOMES (a T-cell regulator) is epigenetically silenced in HCC, impairing immune surveillance. Bacterial dysbiosis may exacerbate these effects by inducing pro-inflammatory cytokines (e.g., TNF-α, IL-6) that activate DNMTs or disrupt methylation pathways.

Intratumoral bacteria play a critical role in hepatocellular carcinoma (HCC) progression by modulating epigenetic and immune microenvironments. This study reveals that *Pseudomonas* activates oncogenes such as *BMI1* and *EZH2* via the LPS-TLR4-NF-κB pathway, driving cancer stem cell traits and chemoresistance. Meanwhile, short-chain fatty acids (SCFAs) produced by *Propionibacterium* may inhibit HDAC activity, indirectly influencing the methylation status of immune-related genes like *EOMES* and impairing CD8 + T-cell antitumor function. *Mycoplasma* disrupts DNMT activity through host genome integration, leading to tumor suppressor silencing. These bacteria reshape the epigenetic landscape of HCC

through metabolites and inflammatory signaling while fostering an immunosuppressive microenvironment, closely linked to poor patient prognosis.

Bacteria exhibit dual roles in HCC: Although *Pseudomonas* abundance is reduced overall in tumors, its residual populations promote invasiveness through epigenetic reprogramming, suggesting strain-specific or microenvironment-dependent effects. This paradox highlights the potential of intratumoral microbiota as dynamic therapeutic targets—for example, combining antibiotics to eliminate pro-tumor bacteria or leveraging probiotics to restore immune surveillance. Future studies should integrate single-strain functional analyses, spatial multi-omics, and clinical cohort validation to elucidate bacterial–host interaction mechanisms and explore microbiome-based personalized therapies, such as microbiota-targeted epigenetic combination strategies.

In summary, our study sheds light on the relationship between Propionibacterium, Mycoplasma, Pseudomonas, and DNA methylation factors that impact survival in HCC. These findings hold clinical relevance and offer valuable insights into possible mechanisms underlying HCC development and progression.

In conclusion, our study has identified *Propionibacterium, Mycoplasma*, and *Pseudomonas* as tumor-specific bacteria in hepatocellular carcinoma (HCC). We have also explored the relationship between the abundance of these bacteria and the expression of DNA methylation genes. Our findings indicate a linear association between the abundance of these bacteria and the expression levels of *BMI1, EOMES, EZH2*, and *SPOCD1*, which are crucial genes in the DNA methylation pathways and have significant associations with HCC overall survival (OS). These findings provide a foundation for further investigating the mechanisms underlying the interactions between intratumoral flora and tumors in HCC.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10620-025-09157-x.

Author Contributions Wei Chen and Xiang Zhang were responsible for the study design, data analysis, and interpretation. Minhui Chi led the experimental work and data collection and played a key role in drafting the manuscript. Qi Zheng supervised the project, assisted in data interpretation, provided critical feedback, and contributed to manuscript revision.

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Data Availability The sequence datasets were released in NCBI PRJNA1006188.

Declarations

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval Informed consent was from participants after explaining the research purpose and procedures. They agreed to anonymize their information. Approval was from the Institutional Review Board of The First Affiliated hospital, Fujian Medical University.

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