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RESEARCH ARTICLE

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The association between gut butyrate-producing bacteria and non-small-cell lung cancer

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Abstract

Background: Recently, it has been found that the gut microbiota may affect the development of lung cancer through the "gut-lung axis." To investigate this relationship, we performed this study to determine whether the gut microbiota in non-small-cell lung cancer (NSCLC) patients is different from that in healthy adults.

Methods: Quantitative PCR (qPCR) was used to detect the expression levels of eight gut butyrate-producing bacteria in healthy adults and NSCLC patients. We enrolled 30 patients with NSCLC and 30 subjects from 100 healthy adults after matching for age and sex.

Results: Compared to healthy adults, most of the gut butyrate-producing bacteria in NSCLC patients were significantly decreased; these included *Faecalibacterium prausnitzii*, *Clostridium leptum*, *Clostridial cluster I*, *Ruminococcus* spp., *Clostridial Cluster XIVa*, and *Roseburia* spp. Among the gut butyrate-producing bacteria, we analyzed *Clostridial cluster IV* and *Eubacterium rectale* were not decreased in NSCLC patients.

Conclusions: We conclude that NSCLC patients had gut butyrate-producing bacteria dysbiosis. Further studies should be performed to investigate the underlying mechanisms of how these specific bacteria affect lung cancer progression and prognosis.

KEYWORDS

butyrate-producing bacteria, dysbiosis, gut microbiota, gut-lung axis, non-small-cell lung cancer

1 | INTRODUCTION

Globally, lung cancer is the most commonly diagnosed cancer (11.6% of the total new cancer cases) and is the leading cause of cancer deaths (18.4% of the total cancer deaths).[1] In China, lung cancer is associated with the highest cancer-related morbidity and mortality.[2] The incidence is about 40/100 000 per year, while the average global incidence is 31.5/100 000 per year.[1] In addition to smoking, air pollution, occupational carcinogenic factors, ionizing radiation, and genetic factors, there are significant changes in the microbiota in lung cancer patients, suggesting that microbiota dysbiosis may also play an important role in lung cancer pathogenesis.[3] However, most of the lung cancer microbiota studies focus exclusively on the lung microbiota as opposed to other microbiota that may be implicated in lung cancer.[4-9] For example, the oral microbiota is associated with the occurrence and development of lung cancer.[10-13] This

mechanism may be related to several potential processes, including microbiota dysbiosis, genotoxicity and virulence effect, metabolism, inflammation, and immune response.[3]

As the most complex microbiota system, the gut microbiota is closely related to innate immunity and adaptive immunity, nutrient absorption, metabolism, tissue development, and inflammatory response. It is well established that gut microbiota influences gastrointestinal cancer.[14] Similarly, extraintestinal cancers are also associated with gut microbiota.[15] In recent years, some studies have reported the relationship between lung cancer and the gut microbiota. Zhang et al found that lung cancer patients had significantly higher levels of *Bacteroidetes*, *Fusobacteria*, *Cyanobacteria*, *Spirochaetes*, and *Lentisphaerae*, but dramatically lower levels of *Firmicutes* and *Verrucomicrobia* than the healthy participants.[16] The study showed that eight predominant genera were significantly different between the two groups.

Studies have suggested that lung cancer patients not only have different gut microbiota, but more importantly, these microbiota affect the therapeutic prognosis of lung cancer. In 2018, a study reported that primary resistance to immune checkpoint inhibitors (ICIs) can be attributed to abnormal gut microbiota composition.[17] Because *Akkermansia muciniphila* is often decreased in lung cancer patients, supplementation with *Akkermansia muciniphila* can improve the ICI response.[17] Another study using the Lewis lung cancer mouse model demonstrated that the commensal microbiota contributes to the anti-lung cancer response, with probiotic co-treatment enhancing the anti-growth and pro-apoptotic effects of cisplatin.[18]

The specific mechanism by which the gut microbiota affects lung cancer is unknown, though some studies indicate that it may be related to immunity.[16-18] One potential mechanism may involve the gut-lung axis. The existence of a lung-gut axis has been suggested more recently, and the basis of this axis theory lies in the "gut-lymph" theory of Samuelson et al.[19] Alterations of this axis have been suggested to result in deleterious consequences, such as pathogen colonization, increased susceptibility to infection, tissue damage, possible development of cancer, and increased mortality.[20,21] With this in mind, it is evident that gut microbiota plays a crucial role in homeostasis in hosts, and that its fine-tuned composition counts for much more than was previously thought.

Therefore, to elucidate this new and very interesting area of research, we conducted a case-control observational study of NSCLC patients. This study consisted of two groups of patients: Group 1 (NSCLC) and Group 2 (healthy volunteers). Transcript expression in the gut microbiota was evaluated by qPCR, focusing exclusively on the differences in butyrate-producing bacteria.

2 | MATERIAL AND METHODS

2.1 | Participant information

This study was approved by the Clinical Research Ethics Committee of First Affiliated Hospital, School of Medicine, Zhejiang University. The study was performed in accordance with the 7th revision of the Declaration of Helsinki (2013), and all participants provided signed, written informed consent. All subjects were ≥18 years old. All the enrolled subjects reported demographic information, including height and weight, sex, age, smoking and drinking habits, lifestyle, and diet. For NSCLC patients, the details also included laboratory tests, tumor pathological type, tumor stage, and tumor site. Exclusion criteria were as follows: history of radiotherapy or surgery; diagnosis of a malignant tumor (except lung cancer); presence of cardiovascular disease (myocardial infarction or stroke); presence of diabetes, hypertension, dyslipidemia, or dementia; activities of daily living (ADL) score <100 points; history of depression; use of probiotics, prebiotics, synbiotics, or antibiotics in the past 4 weeks before enrollment; and history of gastrointestinal surgery. From September 2014 to August 2017, 41 patients with pathological diagnosis of NSCLC were enrolled in the Geriatric ward at the First Affiliated Hospital of Zhejiang University School of Medicine, among which 30 patients were eligible. One hundred healthy adults were enrolled during the same period, and 30 participants were selected for the control group after matching for age and sex.

2.2 | Fecal sample collection, DNA extraction, and PCR

Fresh stool samples from NSCLC patients and healthy controls were collected in anaerobic bags. Samples were divided into five aliquots of 200 mg within 30 minutes after sampling and were immediately stored at −80°C.

2.3 | Detection of butyrate-producing bacteria in the gut by qPCR

All oligonucleotide primers were synthesized by Takara (see Table 1). qPCR was performed using a ViiA 7 real-time PCR system (Applied Biosystems). The amplification was performed using a commercially available kit (TAKARA SYBR Premix EX Taq^{TM}; kit Code: DRR820A): 10 μL SYBR Green PCR premix (Applied Biosystems; Code: 4309155), 0.8 μL of each primer, 2 μL of original template DNA, distilled water to make the final volume to 20 μL. Each reaction was repeated twice, and the error was controlled within 0.5. The amplification temperature and time were as follows: pre-denaturation at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 72°C for 40 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. Fluorescence was measured at 80°C for 10 seconds after the extension step of each cycle to avoid interference from primer dimers, spurious priming, or secondary structure. A final extension step was performed for 5 minutes at 72°C. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by

TABLE 1 Primer sequences and annealing temperatures in this study

Primers	Sequence (5′-3′)	Annealing temperature (°C)
Faecalibacterium prausnitzii	GATGGCCTCGCGTCCGATTAG CCGAAGACCTTCTTCCTCC	58[22]
Clostridium leptum	TTAACACAATAAGTWATCCACCTGG ACCTTCCTCCGTTTTGTCAAC	60[23,24]
Clostridial cluster I	ATGCAAGTCGAGCGAKG TATGCGGTATTAATCTYCCTTT	60[25]
Eubacterium rectale	AAG GGAAGCAAAGCTGTGAA TCGGTTAGGTCACTGGCTTC	61[26]
Clostridial cluster IV	CCTCTTGACCGGCGTGT CAGGTAGAGCTGGGCACTCTAGG	58[25]
Ruminococcus spp.	GGCGGCYTRCTGGGCTTT CCAGGTGGATWACTTATTGTGTTAA	60[27]
Clostridial cluster XIVa	CGGTACCTGACTAAGAAGC AGTTTYATTCTTGCGAACG	55[28]
Roseburia spp.	GCGGTRCGGCAAGTCTGA CCTCCGACACTCTAGTMCGA	60[29]

heating at from 65°C to 95°C at a rate of 0.1°C/s, with continuous fluorescence measurement. The copy number of rDNA operons of these butyrate-producing bacteria in the gut in crude DNA templates was determined by comparing serially diluted plasmid DNA standards run on the same plate. The annealing temperature and sequences for each primer were as previously described and are presented in Table 1.22-29

2.4 | Statistical analysis

All continuous variables were expressed as mean ± standard deviation (SD). The comparison between two groups was performed using the Student's *t* test for independent samples. The categorical variables were tested by chi-squared test. SPSS version 22.0 (SPSS) was used for statistical analysis. Moreover, we evaluated the Spearman's rank correlation between systemic inflammatory markers and gut butyrate-producing bacteria using RStudio version 3.6.1 (RStudio). A value of $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Participant characteristics

A total of 30 patients (20 males and 10 females) comprised the NSCLC group, and 30 individuals (16 males and 14 females) were included in the healthy control group. There were no significant differences in age, sex, BMI, or smoking, and drinking habits between the two groups. In the NSCLC group, 26 patients (86.7%) had adenocarcinoma and 4 patients (13.3%) had squamous cell carcinoma; 24 patients (80%) had peripheral lung cancer, and 6 patients (20%) had central lung cancer; and most of the NSCLC patients (20 patients, 66.7%) belonged to the tumor stage Group I (Table 2).

TABLE 2 Characteristics of the NSCLC and healthy groups

	Healthy group	NSCLC group	*P* value
n	30	30	
Sex (male/female)	16/14	20/10	.292
Age (y)	67.4 ± 6.8	66.0 ± 7.3	.423
Smoking (%)	26.7	46.7	.108
Drinking (%)	23.3	26.7	.766
BMI (Kg/m^2)	23.6 ± 2.7	22.4 ± 2.7	.104
Tumor stage			
I		20	
II		2	
III		5	
IV		3	
Pathological classification			
Adenocarcinoma		26	
Squamous cell cancer		4	
Tumor site			
Peripheral lung cancer		24	
Central lung cancer		6	

Note: The continuous variables are listed as mean ± SD.
Abbreviation: BMI, body mass index.

3.2 | Detection of butyrate-producing bacteria in the gut by qPCR

Gut butyrate-producing bacteria in the two groups were measured by qPCR. First, we performed logarithmic processing of these data, and they were expressed as log10 gene copies in 1 μg fecal DNA; this was followed by comparison using the *t* test. The following bacteria

TABLE 3 Comparison of the logarithmic counts (log10 copies/μg) of some gut butyrate-producing bacteria between the two groups

	Healthy group (n = 30)	NSCLC group (n = 30)	*P* values
Faecalibacterium prausnitzii	7.5 ± 1.1	6.7 ± 1.0	.006
Clostridium leptum	8.0 ± 0.9	7.1 ± 1.0	.001
Clostridial cluster I	5.9 ± 0.8	5.1 ± 1.2	.002
Eubacterium rectale	6.2 ± 1.5	5.4 ± 1.4	.37
Clostridial cluster IV	3.0 ± 1.3	3.0 ± 0.9	.79
Ruminococcus spp.	6.6 ± 1.0	5.7 ± 1.0	.001
Clostridial cluster XIVa	8.0 ± 0.8	6.7 ± 1.6	<.0001
Roseburia spp.	6.6 ± 1.4	5.9 ± 1.1	.035

Note: The continuous variables are listed as mean ± SD.
Abbreviation: NSCLC, non-small-cell lung cancer.

were significantly lower in the NSCLC group compared to that in the control group: *Faecalibacterium prausnitzii* (6.7 ± 1.0 vs 7.5 ± 1.1, *P* = .006), *Clostridium leptum* (7.1 ± 1.0 vs 8.0 ± 0.9, *P* = .001), *Clostridial cluster I* (5.1 ± 1.2 vs 5.9 ± 0.8, *P* = .002), *Ruminococcus* spp. (5.7 ± 1.0 vs 6.6 ± 1.0, *P* = .001), *Clostridial cluster XIVa* (6.7 ± 1.6 vs 8.0 ± 0.8, *P* < .0001), and *Roseburia* spp. (5.9 ± 1.1 vs 6.6 ± 1.4, *P* = .035). However, there were no significant differences in *Clostridial cluster IV* (3.0 ± 0.9 vs 3.0 ± 1.3, *P* = .79) and *Eubacterium rectale* (5.4 ± 1.4 vs 6.2 ± 1.5, *P* = .37; Table 3).

3.3 | Correlation between systemic inflammatory markers and butyrate-producing bacteria

Systemic inflammation-related markers, including white blood cells (WBC), neutrophils, lymphocytes, monocytes, neutrophil-to-lymphocyte ratio (NLR), platelet to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), and C-reactive protein, are potentially independent prognostic factors in lung cancer survival.[16] With the exception of *Roseburia* spp. and *Clostridial cluster IV*, which were negatively correlated with monocytes and WBCs, respectively, all the other tested bacteria were not correlated with the inflammation-related markers (Figure 1).

4 | DISCUSSION

In recent years, studies on the roles of the gut microbiota in extra-gastrointestinal tumors have been increasing,[15,30] and the relationship between lung cancer and the gut microbiota has also been investigated.[16-18] Zhang et al first disclosed the characteristics of the gut microbiota in lung cancer patients.[16] The present study focused exclusively on determining the differences among eight gut butyrate-producing bacteria, which are the most abundant butyrate-producing bacterial species in the gut, using qPCR.[31,32] In addition, these bacteria play an important role in maintaining gut homeostasis by improving gut barrier functions and exerting anti-inflammatory and immunomodulatory effects.[33] There is considerable literature evaluating these eight butyrate-producing bacteria using the PCR technology,[22-29] and thus, this approach is relatively established, ensuring reliability of the results.

We found that compared to healthy individuals, except for *Clostridial cluster IV* and *Eubacterium rectale*, all the other tested butyrate-producing bacteria, including *Faecalibacterium prausnitzii*, *Clostridium leptum*, *Clostridial cluster I*, *Ruminococcus* spp., *Clostridial cluster XIVa*, and *Roseburia* spp., were significantly decreased, indicating that there is significant dysbiosis of butyrate-producing bacteria in NSCLC patients. Several studies[16,34] have demonstrated the presence of gut microbiota dysbiosis in patients with lung cancer, which is consistent with our findings. However, given the differences in the gut microbiota of interest, sampling locations, participant age, and determination methods between our study and previous studies, the specific gut bacterial species in our findings were not the same as those in the other two studies.[16,34] For example, Zhuang et al[34] found that patients with lung cancer have elevated levels of *Enterococcus*. Further, Zhang et al[16] found that lung cancer patients have higher levels of *Bacteroides*, *Veillonella*, and *Fusobacterium* but lower levels of *Escherichia-Shigella*, *Kluyvera*, *Faecalibacterium*, *Enterobacter*, and *Dialister*.

Butyrate-producing bacteria are a type of gut probiotic, which produce butyric acid as their main metabolite. These bacteria exist in the human intestine and can inhibit the growth of harmful gastrointestinal bacteria, promote nutrient absorption, improve intestinal function, and can be beneficial for the overall human health. Butyrate-producing bacteria are also found in the human colon. The most important species appear to be *Faecalibacterium prausnitzii*, *Eubacterium rectale*, and *Roseburia intestinalis* belonging to clostridial clusters IV and XIVa, which belong to the Firmicutes phylum.[35-37]

Butyrate-producing bacteria have received much attention in the past few years because they contribute to intestinal homeostasis by maintaining the intestinal barrier function and exerting immunomodulatory and anti-inflammatory effects.[32,33] Studies have shown that butyrate-producing bacteria are negatively related to irritable bowel disease and colorectal cancer,[38-40] as butyric acid is typically decreased in such patients. The abundance of butyrate-producing bacteria is also lower in patients with metabolic diseases.[41]

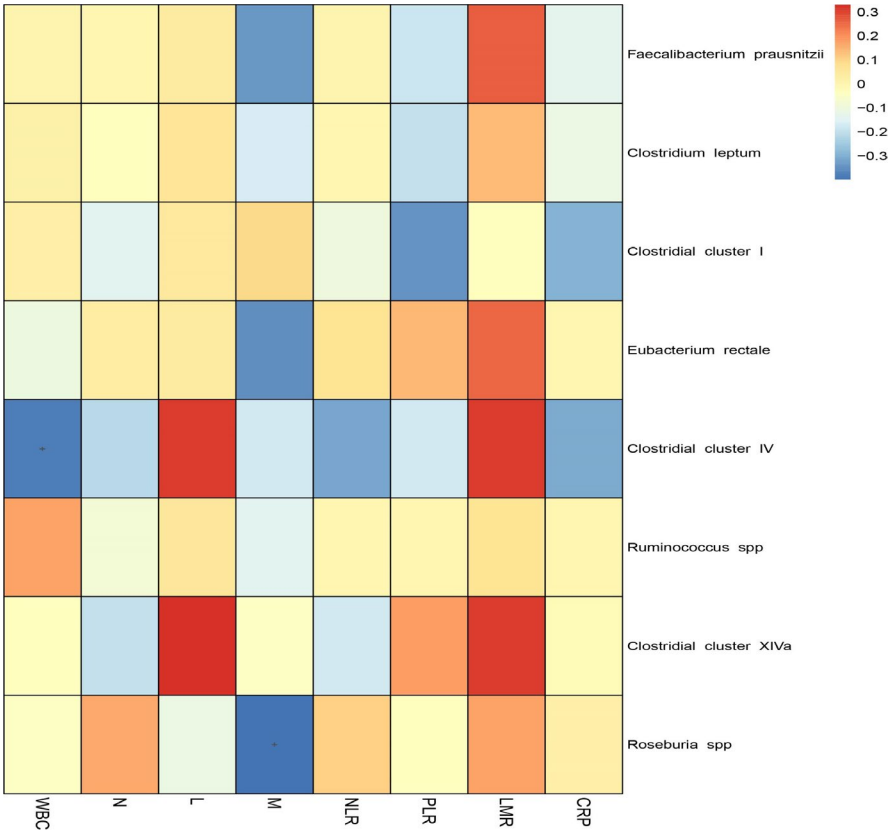

FIGURE 1 Correlations between systemic inflammatory indicators and butyrate-producing bacteria. The Spearman's rank correlation was used to evaluate the statistical importance between systemic inflammatory indicators and the relative abundance of eight butyrate-producing bacteria and was represented by color ranging from blue (negative correlation) to red (positive correlation). CRP, C-reactive protein; L, lymphocytes; LMR, lymphocyte-monocyte ratio; M, monocytes; N, neutrophils; NLR, neutrophil-lymphocyte ratio; PLR, platelet-lymphocyte ratio; WBC, white blood cells; $^{+}P < .05$

As an important product of butyrate-producing bacteria, butyric acid is a major short chain fatty acid (SCFA) preferentially used as an energy source by the gut mucosa.[31] Interestingly, sodium butyrate has shown potent anti-cancer activity as a potential histone deacetylase inhibitor.[42] Particularly in colorectal cancer, sodium butyrate can induce apoptosis of tumor cells.[43] It has also been found that sodium butyrate can inhibit the growth of lung cancer cells. In vitro experiments have shown that sodium butyrate increases the expression of P-glycoprotein in lung cancer cells by upregulation of signal transducer and activator of transcription 3 (STAT3) and stabilization of the ATP binding cassette subfamily B member 1 (ABCB1) mRNA.[44]

To explore the possible mechanisms underlying the decrease in gut butyrate-producing bacteria, we analyzed correlations between systemic inflammatory markers and gut butyrate-producing bacteria and found that most butyrate-producing bacteria were not significantly related to inflammatory markers. We reached a different conclusion from those made in previous studies, which generally indicated significant relationships between butyrate-producing bacteria and inflammatory markers.[45-47] Such other studies[48-51] showed that NLR, LMR, and PLR are associated with poor prognosis in patients with NSCLC. In our study, only two of the eight bacteria were negatively related to WBCs and monocytes, and no bacteria were significantly associated with the other systemic inflammatory markers (such as NLR, PLR, and LMR). Therefore, we could not conclude that butyric acid bacteria influence systemic inflammation. Our findings might be related to the small number of cases or the lack of a causal relationship between systemic inflammatory markers and gut butyrate-producing bacteria in NSCLC.

Recently, many studies have begun to address the relationship between gut microbiota and the lung, which has been referred to as the "gut-lung axis".[19] This theory suggests that the gut microbiota can affect the lungs through local and systemic immune responses. As early as 2015, there were related research studies focused on tumor and gut microbiota.[20,21] In recent years, clinical studies of lung cancer have also confirmed that the gut-lung axis was affected by immunization. In 2018, there were three reports of a relationship between the gut microbiota and ICIs (PD-1/PD-L1).[17,52,53] Combining these highly significant fields of research, we can conclude that gut microbiota is closely related to the immune system, which has a great effect on the efficacy of tumor immunotherapy. Different gut microbiota play different roles in immunity; some "good microbiota" can significantly enhance the efficacy of immunotherapy, whereas "bad microbiota" will not. One of these studies was based on NSCLC patients, which found that the gut microbiota is closely related to the ICI efficacy.[17] Another study showed that some Chinese lung cancer patients with high diversity of gut microbiota showed significantly better response to nivolumab immunotherapy. In terms of progression-free (PR) survival, patients with high gut microbiota diversity can reach 209 days, while patients with low diversity show a PR survival rate of only 52 days.[54]

There are several limitations to our study that should be acknowledged. First, the sample size was small, which might influence

the outcome of this study. Second, we only assessed the relationship between systemic inflammation indicators and gut butyrate-producing bacteria in NSCLC and did not explore whether the gut-lung axis plays an important role in NSCLC progression. Moreover, we did not further measure the inflammatory factors, such as interleukin-12, interleukin-17, and cytotoxic T lymphocyte antigen-4, which might be related to lung cancer, based on results of a previous study.[16] Finally, we did not perform animal experiments to further validate our findings.

5 | CONCLUSIONS

In conclusion, our study confirms a significant correlation between the gut microbiota and lung cancer. Whether lung cancer patients carry a different microbiota or whether the differences in the gut microbiota affect the efficacy of immune-based cancer therapeutics, the gut-lung relationship may be related to the immune system. Although previous studies have found that butyrate-producing bacteria play a certain role in the occurrence and development of tumors, and this is supported by the present study, further research is needed to verify its role in lung cancer. This study was a cross-sectional study, as we did not specifically evaluate the mechanism between gut bacteria and tumor development. In summary, we found that dysbiosis is implicated in NSCLC patients, who have lower levels of gut butyrate-producing bacteria. Further, we found a negative correlation between systemic inflammation markers and gut butyrate-producing bacteria in NSCLC patients. Although we speculate that butyrate-producing bacteria may affect lung cancer through the gut-lung axis, the related mechanism is still unknown. Future studies are warranted to investigate the specific mechanism(s) by which the gut microbiota influences the development of lung tumor.

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