A metagenome association study of gut microbiome revealed biomarkers for chemotherapy efficacy in locally advanced and advanced lung cancer

Zhe Zhao

Chinese Academy of Medical Sciences and Peking Union Medical College

Kailun Fei

Chinese Academy of Medical Sciences and Peking Union Medical College

Zhijie Wang

Chinese Academy of Medical Sciences and Peking Union Medical College

Jianchun Duan

Chinese Academy of Medical Sciences and Peking Union Medical College

Hua Bai

Chinese Academy of Medical Sciences and Peking Union Medical College

Jie Wang (✉ zlhuxi@163.com)

Chinese Academy of Medical Sciences and Peking Union Medical College <https://orcid.org/0000-0002-5602-0487>

Research

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Abstract

Background

Accumulating evidence has confirmed the important role of the gut microbiome in the development and immunotherapy efficacy of lung cancer. However, little is known about the relationship between intestinal flora and chemotherapy. This study investigates the correlation between intestinal flora and chemotherapy efficacy in lung cancer.

Methods

We analyzed baseline stool samples from patients with locally advanced and advanced lung cancer before chemotherapy treatment, through metagenomics of the gut microbiota. The composition, diversity, function, and metabolic pathway analysis of the microbial communities were compared, using the R statistical programming language, among patients with different clinical outcomes.

Results

From September 1, 2018 to September 30, 2019, we obtained stool samples from 64 patients with locally advanced and advanced lung cancer treated with chemotherapy at baseline, consisting of 33 patients who responded to treatment (responders) and 31 patients who did not (non-responders). The median progression-free survival was 7 months (range, 1.5–14.5). *Streptococcus mutans* and *Enterococcus casseliflavus* were enriched in responders ($P < 0.05$), while 11 bacteria including *Leuconostoc lactis* and *Eubacterium siraeum* were enriched in non-responders ($P < 0.05$) by variance analysis. Responders to chemotherapy were associated with significantly higher *Acidobacteria* and *Granulicella*, while *Streptococcus oligofermentans*, *Megasphaera micronuciformis*, and *Eubacterium siraeum* were more abundant in non-responders by Lefse analysis. *Streptococcus mutans* and *Enterococcus casseliflavus* were further identified as bacterial markers for the responders in chemotherapy using unsupervised clustering, and *Leuconostoc lactis* and *Eubacterium siraeum* were the biomarkers for non-responders. Functional analysis of metabolic pathways revealed that the L-glutamate degradation VIII pathway was enriched in responders ($P = 0.014$), and the C4 photosynthetic carbon assimilation cycle, reductive TCA cycle I, and hexitol fermentation to lactate, formate, ethanol, and acetate were enriched in non-responders ($P < 0.05$). In addition, significant associations of bacterial species with clinical indicators such as age, body mass index, and pathological patterns were observed by spearman correlation analysis.

Conclusions

The study showed that the specific gut microbiome of patients with lung cancer might possess a potential predictive role for the clinical outcomes of chemotherapy. Further validation is needed.

Background

Lung cancer is one of the most commonly diagnosed cancers worldwide according to the International Agency for Research on Cancer, causing 2 093 876 (11.6% of all cancers) new cases and 1 761 007 (18.4% of all cancers) deaths in 2018 [1]. More than half of the patients are diagnosed with locally advanced and advanced stages, with a 5-year survival rate of less than 15%. Developing ways to effectively treat lung cancer has always been the top priority of cancer clinical research work. Revolutionary progress of immune checkpoint inhibitors has ushered in a new era of lung cancer treatment [2, 3]. However, immunotherapy only elicits durable responses in 20–30% of patients [4]. Platinum-based systemic chemotherapy is still the cornerstone treatment for patients with locally advanced and advanced lung cancer [5]. Despite continuous progress, the efficacy of chemotherapy is still uneven and lacks predictors. Early identification of patients who are sensitive or resistant to chemotherapy is very important to guide treatment and improve survival. It is therefore clinically significant to develop convenient and noninvasive biomarkers for chemotherapy efficacy prediction.

The human gut microbiome contains nearly 100 trillion interdependent microorganisms that form an ecological balance in quality and quantity under normal conditions [6, 7]. These microorganisms include, but are not limited to, bacteria, viruses, fungi, and protozoa, with bacteria being the most widely studied. The microorganisms participate in the maturation and maintenance of the immunological system, metabolism, and other processes to ensure homeostasis [8], while dysbiosis of the gut microbiome is related to the occurrence and development of multiple diseases. Previous studies focused on the correlation between the gut microbiota and chronic intestinal diseases or the differentiation of the immune system [9, 10]. Recent studies have found that the microbiome can also regulate the development and treatment efficacy of cancers [11–14], and certain metabolites, such as short chain fatty acids and bile acids, are proposed to be critical mediators [15].

Further evidence has demonstrated that the gut microbiota can modulate responses to immunotherapy in lung cancer and might serve as a potential biomarker for immunotherapy efficacy prediction [16, 17]. Non-small cell lung cancer patients with higher baseline diversity of the gut microbiome had better responses to nivolumab. Besides, in the study enrolling forty-two advanced lung cancer patients undergoing programmed death 1 blockade therapy, *Alistipes putredinis*, *Bifidobacterium longum*, and *Prevotella copri* were reported to be enriched in the responders, whereas *Ruminococcus* was overrepresented in nonresponding patients [17]. Another cohort study conducted by Routy et al. showed that the abundance of *Akkermansia muciniphila*, via the recruitment of CCR9+, CXCR3+, and CD4 + T lymphocytes, was positively correlated with immune efficacy in patients with lung cancer and renal cell carcinoma [18].

The efficacy and toxicity of chemotherapy drugs have also been proposed to be regulated by the gut microbiome through multiple ways, including microbiota-mediated biochemical transformation [19]. Gemcitabine, a chemotherapy drug commonly used for lung squamous cell carcinoma, might be degraded by bacterial cytidine deaminase, resulting in poor treatment outcomes [20]. Irinotecan, another

antineoplastic chemotherapy drug, could be converted to toxic metabolites by bacterial beta-glucuronidase [21]. A lung cancer mouse model constructed by Gui et al. showed that normal intestinal flora, such as *Lactobacillus*, may enhance the antitumor effect of cisplatin by increasing serum levels of interferon gamma (IFN- γ) [22]. Previous studies have focused on exploring the role of gut microbes in few individual chemotherapeutic drugs. However, the relationship between the metabolism of most chemotherapeutic drugs and intestinal microorganisms has not been clarified, and neither has the exact role of the gut microbiome in systemic chemotherapy schemes for patients with locally advanced and advanced lung cancer. Moreover, the correlations between gut bacteria and certain chemotherapy prognostic indices have not yet been observed.

Therefore, to help to elucidate this new and extremely interesting field, we conducted a metagenome sequencing analysis of microbiota in stool samples to analyze the correlation between clinical outcomes and the gut microbiome and explore whether specific flora can predict the efficacy of chemotherapy in patients with lung cancer. This work may lay a theoretical foundation for improving the efficacy of chemotherapy by regulating intestinal flora or even flora transplantation in the future.

Methods

Research patients and subjects

The study design was a single-site, correlative study on the effects of the gut microbiome on the efficacy of first-line systematic chemotherapy in locally advanced and advanced lung cancer patients. This study was approved by the Research Ethics Board of the Cancer Hospital of the Chinese Academy of Medical Sciences (Beijing, China), was conducted in accordance with the principles of the Helsinki Declaration, and informed consent was obtained from all subjects.

Locally advanced and advanced lung cancer patients treated with first-line chemotherapy were enrolled in the study between September 2018 and September 2019. The following exclusion criteria were strictly followed: (i) the clinical diagnosis of mental disorders; (ii) a history of gastrointestinal surgery; (iii) a previous diagnosis of a gastrointestinal disease, any autoimmune or metabolic disease; (iv) combined with treatments for other cancers; (v) the occurrence of acute or chronic infections in the past six months; (vi) use of antibiotics, probiotics, or steroids within the past six months. All patients enrolled in our study had a definite histologic pathological diagnosis of lung cancer, for the first time, according to the diagnostic criteria proposed by the 8th edition of the American Joint Committee on Cancer in 2018 [23], and had been prescribed systemic first-line chemotherapy. Additionally, no patients received radiotherapy, targeted therapy, surgery, or immunotherapy for lung cancer before sample collection. Furthermore, patients enrolled had to have measurable lesions according to the response evaluation criteria in solid tumors version 1.1 (RECIST v1.1) [24]. Tumor size was assessed by computerized tomography scan and/or magnetic resonance imaging within 4 weeks before the start of treatment. The treatment strategies for patients enrolled include: (i) pemetrexed combined with cisplatin or carboplatin $\pm$ bevacizumab for patients with lung adenocarcinoma; (ii) paclitaxel or gemcitabine in combination with

cisplatin or carboplatin for lung squamous cell carcinoma; (iii) etoposide in combination with cisplatin or carboplatin for small cell lung cancer; (iv) paclitaxel combined with cisplatin or carboplatin for lung adenosquamous carcinoma. Repeat exams and scans were taken every 2 cycles of chemotherapy. Curative effects were evaluated using the RECIST v1.1 criteria [24], and patients were divided into responders (R) and non-responders (NR) groups according to treatment efficacy. Progression-free survival (PFS) was defined as the interval (in months) from the date of chemotherapy to the date of progression. Moreover, chemotherapy-related bone marrow suppression, nausea, vomiting, and other adverse events throughout the treatment period were recorded according to the National Cancer Institute's common terminology criteria adverse events version 3.0 [25] for each patient.

Samples collection and storage

Stool samples were collected from all patients after diagnosis of locally advanced and advanced lung cancer and before any treatment. All participants had a bland diet and did not smoke or consume alcohol the day prior to sample collection. Fecal samples from all participants were taken at a fresh feces center using a sterile cotton swab, placed in a sterile plastic vial mixed with phosphate-buffered saline, and immediately transferred to a freezer at -80 °C. All samples were stored at -80 °C until further processing.

DNA extraction and sequencing

Fecal bacterial DNA was extracted using the QIAamp PowerFecal Pro DNA Kit (QiaGen, Venlo, Netherlands). Sodium dodecyl sulfate–Tris solution, glass beads (diameter 0.1 mm) (BioSpec), and EDTA-Tris-saturated phenol were added to the suspension, and the mixture was vortexed vigorously by a FastPrep-24 (MP Biomedicals) at 5.0 power level for 30 seconds, with a collection of the supernatant after centrifugation at 20 000 g for 5 minutes. Subsequently, a phenol-chloroform extraction was performed and the supernatant was subjected to isopropanol precipitation. Finally, the DNA was stored at -20 °C. The concentration and purity of the DNA was tested on 2% agarose gels. The amplified DNA was further subjected to library preparation (KAPA HyperPlus PCR-free (96 rxn)) and sequenced on the Illumina MiSeq platform as per the manufacturer's instructions (Illumina technologies, USA).

Data Quality Control

All raw data passed quality control by MOCAT2 and low-quality reads were discarded [26]. Cutadapt software (version v1.14, -m30) was used to remove the sequencing adapter. Then, clean reads were obtained by filtering out low quality reads < 20 or short reads < 30 base pairs (bp) with the SolexaQA package [27]. Finally, SOAPaligner (version v2.21, -M 4 -l 30 -v 10) was applied to get high-quality clean reads for analysis, which were aligned to the human genome (H. sapiens, UCSC hg19) without contaminated host reads [28].

De Novo Assembly

The clean data were assembled by the SOAPdenovo software (version v2.04, an iterative De-Bruijn Graph De Novo Assembler), with parameters as follows: -D 1, -M 3, -L500, for constituting scaffolds of at least 500 bp.

Non-Redundant Metagenomic Gene Catalogue Construction

Genetic structure predictions were carried out with MetaGeneMark [29]. A non-redundant gene catalogue of prediction genes was constructed with CD-HIT [30]. High quality reads were mapped onto the gene catalogue using Burrows-Wheeler Alignment tool for calculation of gene abundance.

Statistical Analyses

MetaPhlAn2 was used to determine the microbial components, including the relative abundance of each level, that is kingdom, phylum, class, order, family, genus, and species [31]. Statistical analyses, such as the composition, diversity, difference, function, and metabolic pathway analyses, were performed using R (version 3.4.3) statistical programming language. Spearman correlation analyses between microbiome and clinical phenotypes were performed with R. Moreover, variation analysis in microbiome between different chemotherapy efficacy groups was identified using Wilcoxon rank-sum permutation test and P value with adjustments according to Benjamini-Hochberg. Non-linear unsupervised clustering analysis was used for further verification. Heatmap showing the unsupervised clustering of the microbiota relative abundance data was performed by ComplexHeatmap in R, in which the cluster_rows and cluster_columns were clustered by Euclidean [32]. After classifying into clusters, we determined the microbiome biomarkers at the species level that showed chemotherapy efficacy combined with clinical efficacy. Finally, we performed gene set enrichment analysis (GSEA) using R, and the genes annotated by the HUMAnN2 gene database were ranked. The gene sets were defined according to the HUMAnN2 pathway for functional analysis of the metabolic pathways.

Results

Patients characteristics

From September 1, 2018 to September 30, 2019, a total of sixty-four patients with locally advanced and advanced lung cancer undergoing definitive chemotherapy were enrolled in the study, provided pretreatment fecal samples, and had follow-up exams and scans. Clinical characteristics, including age, gender, smoking and drinking history, pathological tumor type, clinical stage, and chemotherapy regimen, efficacy, and adverse reactions, were all fully recorded (Table 1). Patients were predominantly male (n = 48, 75%), the median age was 60 years with a range of 33 to 78. Forty-five patients were in advanced stage with different sites of metastases, including bone, brain, pulmonary, pleura, and liver. The pathological types were adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, and small cell lung cancer with a ratio of 34 (53.125%), 10 (15.625%), 2 (3.125%), and 18 (28.125%), respectively. The chemotherapy regimens were pemetrexed + platinum ± bevacizumab for adenocarcinoma, paclitaxel/gemcitabine + platinum for squamous cell carcinoma and adenosquamous carcinoma, and etoposide + platinum for small cell lung cancer. The clinical outcomes were that 33 patients showed RECIST response to chemotherapy, whom we classify as R in this study, and 31 patients did not (NR). In addition, the median progression-free survival was 7 months (range, 1.5–14.5).

Table 1
Clinical characteristics of lung cancer patients who underwent first-line chemotherapy.

Characteristics	No. of Patients (N = 64)	%
Median age at diagnosis (range), y	60 (33–78)	
Median BMI (range), kg/m ²	24.5 (18.7–33.2)	
Gender	64	
Male	48	75%
Female	16	25%
Smoking history	40	62.5%
Drinking history	27	42.19%
Pathology	64	
Adenocarcinoma	34	53.125%
Squamous carcinoma	10	15.625%
Adenosquamous carcinoma	2	3.125%
Small cell carcinoma	18	28.125%
Clinical stage	64	
III	19	29.69%
IV	45	70.31%
Metastasis location	45	
Bone	14	31.11%
Brain	8	17.78%
Pulmonary	19	42.22%
Pleura	16	35.56%
Liver	4	8.89%
Chemotherapy (first-line)	64	
Pemetrexed + platinum ± beacizumab	34	53.125%
Paclitaxel/gemcitabine + platinum	12	18.75%
Etoposide + platinum	18	28.125%

Legend: NR: non-responders to chemotherapy. R: responders to chemotherapy.

Characteristics	No. of Patients (N = 64)	%
Effect	64	
R	33	51.56%
NR	31	48.44%
Median PFS (range), m	7 (1.5–14.5)	
Myelosuppression	64	
0	4	6.25%
I	21	32.81%
II	18	28.125%
III	14	21.875%
IV	7	10.94%
Gastrointestinal reaction	64	
0	6	9.375%
I	40	62.5%
II	14	21.875%
III	4	6.25%
IV	0	0
Legend: NR: non-responders to chemotherapy. R: responders to chemotherapy.		

[Table 1]

No apparent discrepancy in fecal bacterium diversity

We constructed a non-redundant gene set from 64 stool samples. The number of genes in R and NR groups were 1 930 858 and 1 984 255, respectively, of which 1 683 584 were part of the universal gene set (Fig. 1a-b). Species richness, shown by species accumulation curves, indicated that the reads obtained from both groups represented most of the microbiome present in the samples (Fig. 1c). Alpha diversity was determined, by Shannon index, to analyze the complexity of species diversity in each sample. No differences were found in the alpha diversity indices between the R and NR groups (Fig. 2a). In addition, there was no significant difference in beta diversity as constructed by the principal co-ordinates analysis (PCoA) based on the bray-curtis distance of the top several flora species (Fig. 2b), indicating that the primary differences may lie in the less abundant microbiota. The TOP20 microbiome species correlated with the difference between R and NR groups are shown in Table 2, which were

obtained using the similarities percentage method (SIMPER). An additional tabular data file shows this in more detail [see Additional file 1].

Table 2
The TOP20 microbiome correlated with the difference between responders and non-responders.

Species	Average	Sd	Ratio	NR (av)	R (av)	Cumsum
Prevotella copri	0.058	0.088	0.663	9.735	3.708	0.072
Eubacterium rectale	0.049	0.059	0.821	6.040	7.006	0.132
Bacteroides stercoris	0.024	0.037	0.644	2.440	3.553	0.161
Bifidobacterium longum	0.022	0.031	0.695	3.922	1.521	0.188
Faecalibacterium prausnitzii	0.021	0.019	1.132	4.148	4.252	0.215
Bacteroides uniformis	0.021	0.040	0.522	1.725	3.823	0.241
Bacteroides coprocola	0.021	0.035	0.604	2.190	2.695	0.266
Alistipes putredinis	0.020	0.018	1.129	2.841	3.329	0.291
Ruminococcus bromii	0.019	0.026	0.742	2.883	2.043	0.315
Escherichia coli	0.016	0.020	0.778	2.358	1.763	0.335
Bacteroides plebeius	0.015	0.025	0.599	1.429	2.122	0.354
Ruminococcus gnavus	0.015	0.026	0.570	2.215	1.340	0.372
N/A	0.014	0.015	0.943	2.581	1.707	0.388
Pseudomonas aeruginosa	0.014	0.054	0.254	0.006	2.725	0.405
Bacteroides thetaiotaomicron	0.013	0.027	0.503	1.762	1.632	0.422
Bacteroides vulgatus	0.013	0.017	0.772	1.773	2.208	0.438
Bifidobacterium adolescentis	0.013	0.031	0.407	1.731	0.986	0.454
Ruminococcus torques	0.012	0.016	0.789	2.232	1.412	0.469
Roseburia intestinalis	0.012	0.024	0.517	1.558	1.222	0.484
Bacteroides fragilis	0.012	0.020	0.617	2.002	0.960	0.499

Legend: Average: species contribution to average between-group dissimilarity. Cumsum: ordered cumulative contribution, based on item average and sum up to a total of 1. N/A: not applicable. NR (av): average abundances in non-responders. Ratio: average to standard deviation ratio. R (av): average abundances in responders. Sd: standard deviation.

Correlation between gut microbiome and clinical phenotypes

Spearman correlation analyses between microbiome and clinical phenotypes showed that different kinds of clinical manifestations, such as age, body mass index (BMI), pathology, and metastatic sites, were associated with specific flora. Spearman rank correlation coefficients were represented by a heatmap (Fig. 3), with red and blue representing positive correlation and negative correlation, respectively. The results showed that age was inversely related to *Prevotella disiens* ($P < 0.01$) and *Enterococcus gallinarum* ($P < 0.05$); BMI was inversely related to *Clostridium hylemonae* ($P < 0.01$) and had positive correlation with *Streptococcus thermophilus* and *Coprococcus comes* ($P < 0.05$). Patients who reported long-term smoking, were associated with higher abundance of *Campylobacter concisus* ($P < 0.05$) and lower abundance of *Streptococcus thermophilus* ($P < 0.01$) and *Dorea longicatena* ($P < 0.05$). Five species, such as *Dorea longicatena* and *Streptococcus parasanguinis* ($P < 0.001$), were reduced in patients with a long history of drinking. *Collinsella intestinalis* was inversely related to lung adenocarcinoma ($P < 0.05$), while it presented at a higher abundance in patients with small cell lung cancer ($P < 0.05$). *Mitsuokella multacida* ($P < 0.05$) and *Alloscardovia omnicoles* ($P < 0.01$) were enriched in patients with squamous cell lung carcinoma. In addition, baseline metastatic sites also had obvious correlation with different flora. Eleven species, including *Rothia dentocariosa* ($P < 0.001$) and *Solobacterium moorei* ($P < 0.01$), were more abundant in lung cancer patients with pleural metastasis at baseline. *Porphyromonas uenonis* ($P < 0.01$) and three other flora were enriched in patients with pulmonary metastasis. While patients with hepatic metastases had higher abundance of *Pseudomonas mandelii* ($P < 0.001$), *Campylobacter hominis* ($P < 0.001$), and six other species. Moreover, both clinical efficacy and adverse events after chemotherapy were associated with certain bacteria. The enrichment of *Bacteroides nordii* and *Ruminococcus sp_5_1_39BFAA* were associated with severe adverse events after chemotherapy ($P < 0.01$). However, *Gardnerella vaginalis* was inversely related to adverse events ($P < 0.01$). For treatment, *Eubacterium siraeum* ($P < 0.01$), *Leuconostoc lactis* ($P < 0.01$), *Rothia dentocariosa* ($P < 0.05$), and two other flora had negative correlations with efficacy. Besides, *Rothia dentocariosa* showed significant correlations with poorer efficacy and shorter PFS ($P < 0.05$).

Differences in bacterial communities among between different efficacy groups

To characterize the differences in the gut microbial communities between the R and NR groups, we conducted a variance analysis using the Wilcoxon rank-sum permutation test to determine the differences in the relative abundance of flora (Table 3). An additional tabular data file shows this in more detail [see Additional file 2]. The relative abundances of 13 species were significantly different between the two groups. *Streptococcus mutans* ($P = 0.026$) and *Enterococcus casseliflavus* ($P = 0.049$) were enriched in R group, while 11 bacteria, including *Leuconostoc lactis* ($P = 0.002$) and *Eubacterium siraeum* ($P = 0.006$), were enriched in NR group. Significantly different predominant taxa (TOP10) among gut microbiota species were shown in a box comparison plot (Fig. 4a). In addition, the metagenomic

biomarker discovery approach was used to identify the phylotypes responsible for the greatest differences in gut bacteria at the operational taxonomic unit level for distinguishing between the R and NR groups by Lefse analysis, showing that the responders to chemotherapy were associated with significantly higher levels of *Acidobacteria* and *Granulicella*. While *Streptococcus oligofermentans*, *Megasphaera micronuciformis*, and *Eubacterium siraeum* were more abundant in non-responders (Fig. 4b-c).

Table 3
Difference in relative abundance of microbiome species between responders and non-responders (TOP 20).

Species	NR (av)	R (av)	W	P value	Padj
Leuconostoc lactis	0.012	0	643.500	0.002	0.715
Eubacterium siraeum	0.454	0.115	693.500	0.006	0.715
Butyrivibriocrossotus	0.603	0	610.500	0.009	0.715
Candidate division_TM7_single_cell_isolate_TM7b	0.002	0	594	0.018	0.715
Megasphaera micronuciformis	0.064	0.004	632	0.022	0.715
Solobacterium moorei	0.006	0.001	648	0.026	0.715
Streptococcus mutans	0	0.006	434	0.026	0.715
Rothia dentocariosa	0.004	0.0003	621	0.032	0.715
Erysipelotrichaceae bacterium	0.013	0.001	633	0.033	0.715
Granulicatella elegans	0.003	0.0001	598	0.033	0.715
Turicibacter sanguinis	0.005	0	577.500	0.036	0.715
Streptococcus oligofermentans	0.001	0	577.500	0.036	0.715
Enterococcus casseliflavus	0	0.003	449.500	0.049	0.715
Roseburia inulinivorans	0.347	1.727	366	0.051	0.715
Citrobacter koseri	0.0001	0.002	434	0.056	0.715
Lactobacillus johnsonii	0.003	0.001	604	0.060	0.715
Clostridium hylemonae	0.009	0	561	0.072	0.715
Bacteroides intestinalis	0.138	0.140	637	0.078	0.715
Streptococcus cristatus	0.004	0.001	609	0.079	0.715
Bifidobacterium longum	3.922	1.521	642.500	0.080	0.715

Legend: NR (av): average abundances in non-responders. P_{adj}: p-values adjusted using the Benjamini-Hochberg method. R (av): average abundances in responders. W: Wilcoxon test statistic.

Identification of significant taxa clusters at the species level as bacterial markers for the efficacy of chemotherapy using unsupervised clustering

The discrepancies at the species level were comprehensively assessed by the deconvolution of the metagenome data. Unsupervised clustering was performed using `cluster_rows` and `cluster_columns` with euclidean by ComplexHeatmap in R, which classified the species into 5 clusters. Two additional tabular data files show these in more detail [see Additional file 3 and 4]. Finally, a heatmap based on the unsupervised hierarchical clustering displayed species differences between individuals (Fig. 5). Comparing the clustering data with the treatment efficacy for each patient allowed for the identification of significant taxa clusters at the species level as bacterial markers for chemotherapy efficacy in locally advanced and advanced lung cancer patients. *Streptococcus mutans* and *Enterococcus casseliflavus* were significantly enriched in the R group, consistent with the result of variance analysis, and can be used as biological biomarkers for efficacy of chemotherapy. On the other hand, *Leuconostoc lactis* and *Eubacterium siraeum* were the bacterial markers for the NR group, whose P values also consistently showed statistical significance.

Differences in metabolic pathway analysis for functional analysis

To fully understand the differences in the metabolic networks between the R and NR groups, all the metabolites with differential expression were submitted to HUMAnN2 for metabolic pathway enrichment analysis. An additional tabular data file shows this in more detail [see Additional file 5]. Three metabolic pathways were enriched in NR group, including “PWY-241: C4 photosynthetic carbon assimilation cycle, NADP-ME type” ($P < 0.001$), “P23-PWY: reductive TCA cycle I” ($P = 0.007$) and “P461-PWY: hexitol fermentation to lactate, formate, ethanol, and acetate” ($P = 0.025$). While the metabolic pathway called “PWY-5088: L-glutamate degradation VIII (to propanoate)” was abundant in the R group ($P = 0.014$). Additionally, we calculated the metabolite potential based on the relative abundance of the metabolic microflora producing or digesting metabolites and conducted a variation analysis. The heatmap of significantly different metabolites is shown in Fig. 6, and the results suggested that the aliphatic acid or carbohydrate pathways may be used to distinguish between the two groups.

Discussion

The gut microbiome may be a modifiable factor that affects cancer treatment efficacy and toxicity [33]. In the present study, we characterized the composition and differences in the gut bacteria associated with different chemotherapeutic outcomes from 64 lung cancer patients. This is the first detailed report of human gut microbiome metagenomic profiling in lung cancer patients treated with first-line chemotherapy. In this study, we have demonstrated the correlation of gut microbiota with clinical

phenotypes in lung cancer patients and identified specific microbial candidates that might contribute to predicting the chemotherapy efficacy. These findings provided a broader understanding of the effect of the gut microbiome on chemotherapy efficacy in lung cancer patients, paving the way for further investigation in this research area.

Our results showed that the abundance of *Prevotella disiens* and *Enterococcus gallinarum* declined with age. Previous studies have shown that the translocation of gut microflora, such as *Enterococcus gallinarum*, to systemic tissues triggers intense autoimmune responses [34]. However, the correlation between the abundance of these species and age was first proposed in this study, which deserves to be further investigated. Smoking is a recognized risk factor for lung cancer, whose pathogenic mechanism has been extensively studied [35]. The abundance of *Streptococcus thermophilus* was obviously reduced in patients with a history of long-term smoking. *Streptococcus thermophilus* possess in vitro probiotic properties along with anticancer activity [36]. Therefore, taking into consideration previous microbiome studies and our correlation analysis, we recommend quitting smoking to maintain the abundance of beneficial bacteria. Different pathological types of cancer appear to have their own unique gut microbial characteristics [37]. In our study, lung cancer patients with different types of pathology also showed distinct microbial signatures. For example, *Collinsella intestinalis* was reduced in lung adenocarcinoma, while enriched in small cell lung cancer, and *Mitsuokella multacida* and *Alloscardovia omnicolens* were enriched in squamous cell lung carcinoma. These differences may help distinguish the pathological cancer type of patients who do not have adequate conditions for puncture biopsy diagnosis. Several microbes such as *Blautia obeum* and *Akkermansia muciniphila* have been proved to be increased only in metastatic lung cancer patients [37]. Our study further reveals the differences in the flora associated with different metastatic sites (Fig. 3), which may lay the foundation for the individual management of patients.

Data from a previous report has suggested that the treatment effects, including chemotherapy or a combination of chemotherapy with immunotherapy, in cancer patients are positively correlated with some specific types of gut microbiota, such as *Bacteroides ovatus* and *Bacteroides xylanisolvens* [38]. However, the correlation between the efficacy of chemotherapy in lung cancer patients and certain specific flora is still unclear. In our study, we proposed microbial biomarkers to predict the efficacy of chemotherapy in patients with locally advanced and advanced lung cancer for the first time. Through correlation analysis, variance analysis, and unsupervised clustering, we gradually determined the gut microbes that might predict the efficacy of chemotherapy in lung cancer patients. Our results showed that *Streptococcus mutans* and *Enterococcus casseliflavus* were biomarkers for better chemotherapy outcomes. *Streptococcus mutans* is an organism from carious lesions, whose natural habitat is the human oral cavity, with possible translocation to other tissues [39]. The production of glycan, a hydrolytic proteoglycan, by this microbe gives it the ability to adhere to epithelial cells, which can affect the cell–cell adhesions of the host, by influencing the level of functional E-cadherin at the cell–cell border, and enhance the process of tumor cell dissociation and invasion [40]. However, there are no studies on the impact of treatment efficacy. Interestingly, in our study, *Streptococcus mutans* was found to be enriched at baseline in patients with better outcomes, indicating that it may potentially contribute to chemotherapy

efficacy in lung cancer. Gut microbiota, including *Enterococcus*, has been found to be significantly higher in cancer patients, especially those with colorectal cancer, than in healthy people, demonstrating the relevance of this flora to cancer [41]. Moreover, *Enterococcus* is more abundant in metastatic melanoma patients that responded to immunotherapy [42], which may lead to improved tumor control and greater efficacy of immunotherapy by augmented T cell responses. In our study, *Enterococcus casseliflavus* was also shown to be related to better chemotherapy efficacy in lung cancer. Therefore, the combination of *Streptococcus mutans* and *Enterococcus casseliflavus* seemed to be biomarkers for greater chemotherapy efficacy. On the other hand, *Leuconostoc lactis* and *Eubacterium siraeum* were the bacterial markers associated with poor chemotherapy effect. *Leuconostoc* bacteria were initially considered for a wide range of uses in the food industry due to their fermentation properties and odor-producing compounds [43]. In recent years, the role of probiotics in anti-tumor therapy has been explored [44]. *Leuconostoc mesenteroides* isolated from traditional dairy products promoted apoptosis in colon cancer by regulation of MAPK1, AKT, NF- κ B, and some key oncomicroRNAs [44]. Interestingly, we found another species, *Leuconostoc lactis*, that had a negative effect on lung cancer chemotherapy, which contradicts the probiotic characteristics of this genus shown in previous studies, and is worth being explored in depth. *Eubacterium rectale* seemed to be more abundant in healthy persons than in patients with prostate cancer [45]. However, the relationship with anti-tumor therapy has not been investigated. *Eubacterium siraeum* was negatively correlated with chemotherapy efficacy for lung cancer patients in our study, which poses a new challenge for the role of this flora in anti-tumor therapy. Overall, we proposed that enrichment of *Leuconostoc lactis* and *Eubacterium siraeum* represented a biomarker for poorer chemotherapy efficacy in lung cancer. Finally, the detailed molecular mechanism for enhancement of chemotherapy efficacy by any of the bacteria discussed in this study remains unknown, which needs exploring with in-depth experiments in a mouse model.

Taking the challenges of the lability of mRNA and difficulty in standardized sample collection for metatranscriptomics into consideration, we have attempted a metagenomic functional pathway analysis to gain functional insight into changes in the gut microbiome. However, the enriched or reduced abundance of genes in a certain microbiome is still difficult to explain. As studied previously, anacardic acid had an antitumor effect, mediated by enhanced T-cell recruitment, in several preclinical models [46]. Similarly, based on the analysis of metabolic pathways and metabolites in our study, hexitol fermentation and the aliphatic acid or carbohydrate pathways may be reasonable potential targets for metabolic intervention combined with chemotherapy.

Our study had some limitations. First, the sample size of lung cancer patients treated with chemotherapy was relatively small. Therefore, we could not comprehensively and systematically profile the microbial biomarkers for chemotherapy efficacy in lung cancer patients. Thus, a larger number of individuals is needed to verify our findings. Second, we did not monitor the dynamic bacterial community structure of patients during chemotherapy, which might lead to a better understanding of the alterations in gut bacteria associated with chemotherapy. Therefore, further research on this subject is required, and studies with a longitudinal design using the lung cancer animal model treated with chemotherapy drugs

to investigate the underlying mechanisms of the relationship between gut bacteria and chemotherapy efficacy in lung cancer are preferable.

Conclusions

In conclusion, we present the description of the gut microbiota in patients with different chemotherapy outcomes, providing a significant first step in understanding the relationship between the gut microbiome and chemotherapy efficacy in lung cancer. Our work not only extends this observation to monitoring the therapeutic response in lung cancer patients treated with chemotherapy, but also may facilitate clinical therapeutic strategies from a microbial perspective.

Abbreviations

BMI

body mass index

Bp

base pairs

IFN- γ

interferon gamma

NR

non-responders

PCoA

principal co-ordinates analysis

PFS

progression-free survival

R

responders

RECIST

response evaluation criteria in solid tumors

Declarations

Ethics approval and consent to participate

All procedures were performed in accordance with and followed the ethical standards of the Human Laboratory Responsibility Committee of the Cancer Hospital of the Chinese Academy of Medical Sciences (Beijing, China) and complied with the 2013 revised Declaration of Helsinki. Informed consent was obtained from all patients enrolled in the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ZZ collected the patients' stool samples acquisition, recorded the patient information, analyzed and interpreted the patient data regarding the gut microbiota and chemotherapy efficacy. ZZ was a major contributor in writing the manuscript. KLF collected the patients' stool samples acquisition and participated in the analysis and interpretation of data. ZJW analyzed and interpreted the data. JCD arranged the review and evaluated the chemotherapy efficacy of patients. HB designed and drafted the work, analyzed and interpreted the data. JW designed and substantively revised the work, programmed the statistical analysis and interpreted the comprehensive data. All authors read and approved the final manuscript.

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Figures

Figure 1

The number of sequenced genes and species accumulation curve in responders and non-responders. The Wayne diagram shows the non-redundant metagenomic gene catalogues constructed by high quality reads in the responder and non-responder groups, presented in a pie chart (a) and a box comparison chart (b). Species accumulation curve, with a tendency to gradually flatten out, was projected using vegan in R programming language (c).

Figure 2

Alpha and beta diversity between responders and non-responders. Boxplots showing the alpha diversity were evaluated by Shannon index using vegan in R programming language (a). Principal coordinates analysis (PCoA) revealing the beta diversity for responders and non-responders were exhibited with bray-curtis distance (b). The first two principal coordinates (PCs) were labeled with the percentage of variance explained (12.7 % and 9 %).

Figure 3

Heatmap of associations between bacterial species and clinical phenotypes. Heatmap shows the correlation between the abundance of bacterial species and different clinical parameters, in which the columns represent various clinical phenotypes and the rows represent species. Spearman rank correlation coefficients were represented by the heatmap, with red, blue, and white indicating perfect positive, negative, and no correlation, respectively. Color of boxes indicates correlation coefficient (r) values, with darker colors indicating greater relevance. Spearman's correlations were employed in agreement with data distribution, verified by Shapiro-Wilk test. Asterisks (*) in the heat map cells indicate the P value for that correlation. Significant correlations with * ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$).

Figure 4

Differences in the microbiomes of patients with different chemotherapy efficacy outcomes. Significant differences in relative abundance of the predominant taxa among gut microbiota species between responders and non-responders were projected as box comparison charts (a). Differential taxonomic abundance between responders and non-responders (b, c) were analyzed by linear discriminate analysis coupled with effect size measurements (Lefse) as a histogram (c) and a cladogram (b). The listed bacterial floras were significantly ($P < 0.05$, Kruskal-Wallis test) gathered for their respective groups.

Figure 5

Heatmap of species differences between individuals based on unsupervised hierarchical clustering. Unsupervised hierarchical clustering was applied to draw the heatmap, with red, blue, and white indicating enrichment, reduction, and no correlation, respectively, which showed the microbial species abundance was different between the responders (Effect = 1, red dot) and non-responders (Effect = 0, green dot). Color of boxes indicates relative abundance, and darker colors indicate greater abundance.

Figure 6

Heatmap of significantly different metabolites responders and non-responders. Heatmap shows differentially abundant metabolites between responders and non-responders, in which columns represent samples (green = responders, purple = non-responders) and rows metabolites. The heatmap visualization is used to encode individual abundance of the metabolites for each sample as colors (red, relative enrichment; blue, relative reduction; white, no correlation). Designation of metabolites were indicated on the right hand-side of the figure.

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